Improved production and purification of recombinant proteins from mammalian expression systems

Thesis submitted for the degree of

Doctor of Philosophy

By

Alexander William Kinna

Supervised by Dr Darren Nesbeth and Professor Kerry Chester

Department of Biochemical Engineering, University College London, Torrington Place, London, UK

Department of Oncology, UCL Cancer Institute, Paul O'Gorman Building, University College London, 72 Huntley Street, London, UK

Declaration

I, Alexander Kinna, confirm that the work presented here is my own. Where information has been derived from other sources, I confirm that this has been indicated within the thesis.

Signature ……

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Abstract

The biopharmaceutical industry is becoming increasingly reliant on recombinant proteins as therapeutic agents. The work presented here details the development of rapid mammalian expression systems and novel capture methods for use in early recombinant protein development.

A key aim was to investigate expression of recombinant proteins via cost effective production methods and to compare the resultant products at small scales of manufacture. A model single chain variable fragment Fc conjugate (scFv-Fc) targeted against a clinically relevant glycoprotein, the carcinoembryonic antigen (CEA), was expressed and characterised using both transient and stable-based expression of transgenic DNA. Transient gene expression in suspension HEK293 cells produced a maximum scFv-Fc level of 72μg/mL, which was used for initial protein characterisation. A stable pool of transfected CHO cells was also generated using a ubiquitous chromatin opening element (UCOE)-based vector achieving increased protein expression and culture periods when combined with a fed-batch regimen.

Characterisation of the resulting proteins showed that stability and effector function was maintained across transient and stable production methods at all scales, indicating that preliminary data generated from transient expression in HEK293F cells could be generalised to predict that of protein stably expressed in CHO cell populations.

A significant bottleneck in harvesting and purifying proteins from cell containing feed streams is the requirement for solid-liquid separation prior to capture. Therefore, a technique was proposed for direct capture of recombinant protein from unclarified feed streams that can integrate directly into the bioreactor harvest line. The process was demonstrated using immobilised metal affinity chromatography (IMAC) capture of recombinant CEA with a polyhistidine (His^6) tag from a bioreactor culture. This provides a basis for direct primary capture of recombinant proteins from unclarified mammalian cell feed streams that could be generalized to other capture methods and proteins.

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Ab: Antibody

ADCC: Antibody dependent cell-mediated cytotoxicity

CDC: Complement dependent cytotoxicity

CDR: Complementarity determining region

cDNA: Complimentary DNA

CEA: Carcinoembryonic Antigen

CFU: Colony forming unit

CHO: Chinese hamster ovary cell

COG: Cost of Goods

DNA: Deoxyribonucleic acid

DMEM: Dulbecco's modified Eagle medium

DHFR: Dihydrofolate reductase

DSC: Differential scanning calorimetry

DSF: Differential scanning fluorimetry

EGFR: Epidermal growth factor receptor

ER: Endoplasmic reticulum

Fab: Fragment antigen-binding

Fc: Fragment crystallisable

FCS: Foetal calf serum

FPLC: Fast protein liquid chromatography

FRT: flp recombinase targeting motif

GFP: Green fluorescent protein

GS: Glutamine synthetase

h: Hours

HAMA: Human anti-mouse antibody

Hc: Antibody heavy chain

HEK293: Human embryonic kidney cell

HIS4: Histidinol dehydrogenase

HIS: Histidine 6 repeat tag

Ig: Immunoglobulin

LDH: lactate dehydrogenase

Lc: antibody light chain

mAb: Monoclonal antibody

min: Minutes

mRNA: messenger RNA

NHL: Non-Hogdkin's lymphoma

N-Glyc: N-linked glycosylation

OD: Optical density

O-Glyc: O-linked glycosylation

O-Man: O-linked mannosylation

PAGE: Polyacrylamide gel electrophoresis

PCR: polymerase chain reaction

Penstrep: Penicillin streptomycin

PMT: Protein mannosyl transferase

PTM: Posttranslational modification

*q*P: Single cell productivity

RAG: Recombination activating genes

RFP: Red fluorescent protein

s: Seconds

scFv: Single chain variable fragment

scFv-Fc: Single chain variable fragment Fc conjugate

SDS: Sodium dodecyl sulphate

Sub2: Subtilisin

SSI: Site-specific integration

TGE: Transient gene expression

TGF-β: Transforming growth factor beta

U: Cell density

UK: United Kingdom

XBP: X-box binding protein

Chapter 1 Introduction

1.1 Recombinant proteins as therapeutics

Proteins are macromolecules formed of amino acid chains folded into three-dimensional structures. They play a dynamic role in almost every cellular function from receptor signalling and motility to catalytic reactions and cellular differentiation (Leader et al., 2008).

This functional variety of protein activities and the advent of recombinant DNA technology have combined to provide vast opportunities for the commercial development of recombinant proteins as production means become more efficient. Enzymes are now used routinely for biocatalytic reactions in industry enabling their use in everything from agriculture and bioremediation to medicine and food preparation (Demain and Vaishnav, 2009). Further exploitation and manipulation of recombinant protein structure and function for therapeutic uses has brought about the rise of the biopharmaceutical industry.

Some therapeutic proteins can be purified and utilised in their natural state from donors e.g. pancreatic enzymes or human growth hormone, or alternative sources such as native proteins from plants and microbes. However isolation of protein coding genes via recombinant DNA technology has facilitated movement of DNA into transgenic host cells for improved production. Transgenic expression of these proteins achieves reduced associated costs and improves the safety and quality of the product. Molecular and synthetic biology has also allowed the precise engineering of DNA sequences in order to create novel therapeutic proteins targeted for specific indications.

The list of licensed recombinant therapeutics currently includes hormones, enzymes and immunoglobulins amongst others. The year on year sales of biologics has outpaced many predictions as technology has advanced enabling improved protein engineering, product safety and yield (Figure 1.1 Growth in sales of biologics and antibodies in the US from 2002-2012. **Data collated from (Aggarwal, 2014, 2012, 2011, 2010, 2008,; Chames et al., 2009; Dimitrov and Marks, 2009).**

Monoclonal antibodies (mAbs) and antibody derivatives in particular have emerged as a class leader in the treatment of many diseases, with successes in oncology and autoimmune disorders. In 2012 the market for biologics was $63.6 billion, of which antibodies generated $24.6 billion (Elvin et al., 2011, Aggarwal, 2014). The number of potential targets for immunotherapy is ever increasing thanks to improved understanding of disease (Pillay et al., 2011) and the FDA approved biologics list has seen a rapid increase over the last 10-15 years with a total of around 90 now in use (Kinch, 2015). Still, biologics development continues at a rapid rate with commercial pipelines around the world highly dependent on them for new growth. The percentage increase of biologics and antibody sales have closely followed each other over the last 10 years with 70% and 78% increases in sales respectively.

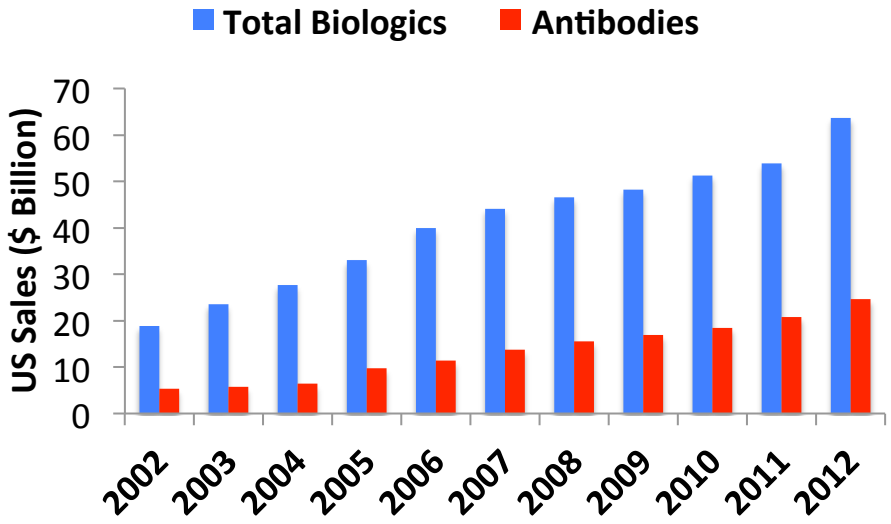


Figure 1.1 Growth in sales of biologics and antibodies in the US from 2002-2012. Data collated from (Aggarwal, 2014, 2012, 2011, 2010, 2008,; Chames et al., 2009; Dimitrov and Marks, 2009).

Whilst the general use of recombinant therapeutic proteins has increased, the time and costs associated with their development remains exceptionally high, primarily due to the challenges encountered during rapid expression and characterisation of large numbers biologics from host systems and the high risk of product failure at clinical stages.

Microbial expression systems have significant advantages over mammalian cells for expression and processing purposes, however mammalian expression of complex proteins remains a costly necessity adding significant challenges to the industry.

Improved mammalian cell specific productivity, feeding strategies and protein engineering have combined to produce cell densities in excess of $1x10^7$cells/mL and yields of up to 8g/L (Farid, 2007; Hacker et al., 2009). However these advances have in turn created new challenges for downstream processing, leading to a focus on development of high capacity methods for the primary capture of protein from high cell density feeds.

1.2 Antibodies

1.2.1 Structure and function of antibodies

The immunoglobulin protein superfamily contains many proteins that share subunit structures and binding properties including cell surface receptors and soluble proteins. Antibodies are immunoglobulins that consist of two polypeptide chains of 55 kDa each and two of 22 kDa known as the heavy and light chains respectively. Disulphide bridges and non-covalent bonds hold these chains together forming a Y-shaped protein of approximately 155 kDa (Figure 1.2 Antibody IgG1 Structure).

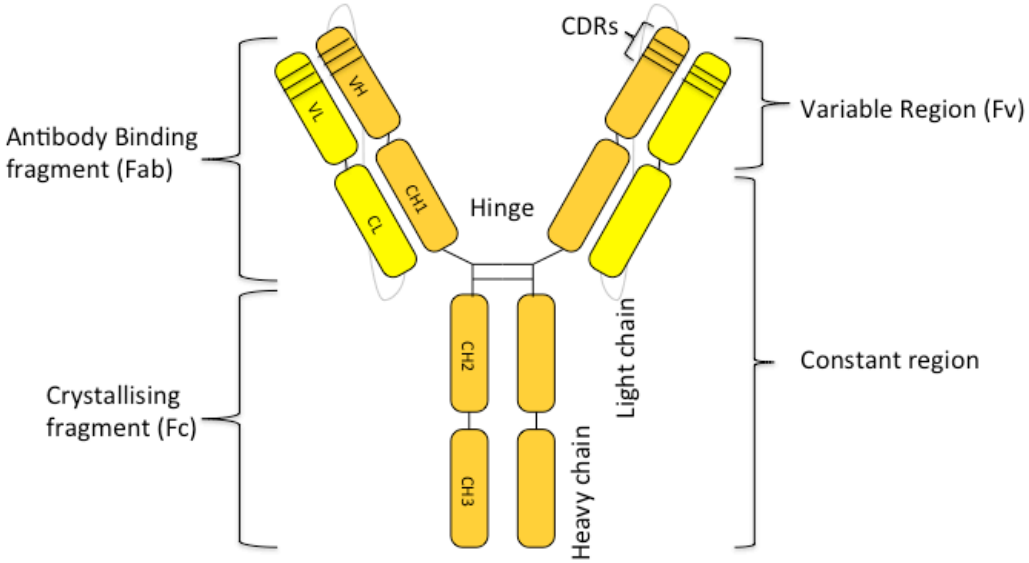


Figure 1.2 Antibody IgG1 Structure. Two light and two heavy chains of 22 kDa and 55 kDa respectively make up a whole antibody. These can be separated into the Fab and Fc regions that are linked by the hinge region. The three complementarity determining regions are located in the variable region of each chain (adapted from Natsume et al., 2009).

The molecule can be divided into two regions as characterised by observed fragmentation after proteolytic cleavage by the enzymes papain and pepsin (Porter, 1959; Nisonoff et al., 1960). Cleavage via papain produces a set of three fragments, two of which display antigen-binding properties (Fragment Antigen Binding- Fab) and contain highly variable regions responsible for specificity of an antibody to its epitope (Jones and Gellert, 2004). The third fragment is termed the Fc or Fragment crystalline after its crystallisable structure. Antibody isotypes can be classified into

five groups (IgG, IgM, IgA, IgE and IgD) depending on the type of heavy chain constant regions.

1.2.2 Glycosylation of recombinant proteins and antibodies

Glycosylation of recombinant proteins is the covalent addition of an oligosaccharide sugar group to specific amino acid residues, occurring in the endoplasmic reticulum and Golgi apparatus. Interactions between oligosaccharides via hydrogen bonding contribute to the overall folding and structure of the protein, hence glycosylation affects many properties including electrical charge (pI), solubility, mass, size and effector function (Jefferis, 2012, 2008).

Oligosaccharide attachment can be in either N-linked or O-linked forms. N- linked glycosylation is the attachment of branched mannose and glucose containing oligosaccharides to an asparagine in the amino acid sequence asp-X-ser/thr. In mammalian cells enzymatic removal of various residues from the high mannose structure enables protein folding, with assistance from chaperones, before further reduction of the oligosaccharide and transit to the Golgi apparatus where final adjustments form a complex diantennary structure (Figure 1.3 Glycosylation of antibody CH2 domainand **Figure 1.5 Examples of yeast, plant, insect and human glycosylation structures**). N-linked additions can be extended in a branched fashion whilst O-linked glycosylation is extended in a single chain at serine or threonine residues and the oligosaccharide chains are capped by sialic acid or phosphate group addition. For a full review of antibody glycosylation structure and mechanism see Jefferis, 2008.

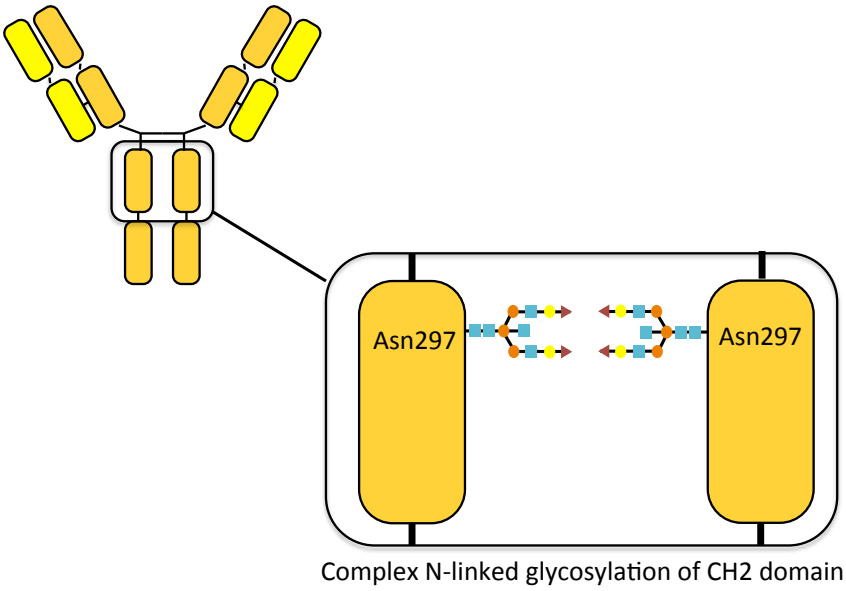


Figure 1.3 Glycosylation of antibody CH2 domain. Correct glycosylation of antibody CH2 domains at Asn297 is required for proper effector function and to reduce immune response in patients.

1.2.3 Effector function

Antibodies function as immune effector proteins with specific affinity for molecular targets known as antigens based on their variable domain CDR regions. The Fc region has multiple effector functions enabling coupling of antigen recognition and immune response as well as moderating pharmacokinetics (Zalevsky et al., 2010). Once bound to an antigen, antibodies can stimulate the innate immune system to destroy the target either via antibody dependant cell-mediated cytotoxicity (ADCC) or by a system of protein cascades known as complement dependant cytotoxicity (CDC) (Gelderman et al., 2004). ADCC involves initiation of cytotoxic action against targets via Fc binding to FcγR (CD16) receptors on cytotoxic cells called natural killers to induce degranulation (Woof and Burton, 2004). These innate properties enable the use of antibodies as therapeutic agents to target a number of diseases.

1.2.4 Therapeutic applications

Paul Ehrlich originally described an ideal 'magic bullet' therapy that could directly bind to a single specific target in the early 1900's. Kohler and Milstein realised this concept in 1975 by pioneering hybridoma technology allowing the identification, isolation and *in vitro* culture of a clonal population of antibody-producing B-cells.

Hybridoma technology uses a mouse model to produce antibodies of interest after exposure to an antigen. Once an immune response has developed, differentiated B-cells can be removed and fused to an immortalised myeloma cell line to produce a clonal hybridoma that can be cultured *in vitro* (Köhler and Milstein, 1975). Hybridoma cells can then be selected via screening for expression of the desired antibody.

During the 1980's the first monoclonal antibodies (mAbs) were developed for treatment of cancerous diseases, allowing highly specific targeting of molecules on cells, known as markers. Early *in vitro* and *in vivo* experiments with mAbs were encouraging, however the results in human patients were less spectacular. Clinical trials resulted in immunogenic responses as patients developed Human Anti-Mouse Antibodies (HAMA) resulting in rapid clearance from the body and reduction in efficacy (Tjandra et al., 1990). The need to make antibodies less recognisable to the immune system was established by the reduction in immune system activity in response to humanised antibodies (Mirick et al., 2004). Antibody engineering

Recombinant DNA technology enabled manipulation of genetic regions to construct and combine regions of antibodies into new proteins. The HAMA response was over come by replacing mouse constant regions with those of a human antibody creating a chimeric molecule that reduced the exposure of foreign regions to the immune system.

Biogen IDEC (Massachusetts, USA) produced the first chimeric antibody, Rituximab, which was licensed by the FDA in 1997. Rituximab is a mouse-human chimeric antibody targeted at CD20 for B-cell destruction, a therapy for non-Hodgkin's

lymphoma and other autoimmune disorders (McLaughlin et al., 1998; Reff and Heard, 2001).

Further advances in humanisation of antibodies include CDR grafting, where the affinity defining CDR regions from mouse antibodies are grafted onto a human antibody scaffold leaving a final product with only 5-10% murine sequence compared to around 65% in chimeric antibodies (Winter and Harris, 1993). The first fully humanised antibody was trastuzumab from Roche in 1998, an anti-HER2 breast cancer treatment, that has since become one of the most successful treatments available (Bryant, 2006).

Therapeutic applications of monoclonal antibodies now include the recruitment of the immune system for cytotoxic action, receptor and ligand interaction blocking and as vehicles for drug delivery (Reff and Heard, 2001; Berger et al., 2002; Filpula, 2007; Weiner et al., 2010; Alley et al., 2010; Beck et al., 2010; Pillay et al., 2011; Redman et al., 2015). Conjugations of mAbs to cytotoxic, enzymatic or radiolabelled drugs maximises target specific treatment whilst reducing non-specific cell death (bystander effect). A table containing examples of conjugated antibodies can found in Parakh et al., 2016 and Scott et al., 2012.

Aside from their use in therapeutic applications, antibodies have been used in research to track, detect, label and alter targets. However the majority of research purposed antibodies are polyclonal, originating from a range of B-cells after animal immunisation or from hybridoma cells often leading to irreproducible data. Hence, there is a concerted effort amongst the research community to use recombinant antibodies, which are fully characterised and sequence defined where possible (Bradbury and Plückthun, 2015). This will have a significant effect on the number of recombinant antibodies in use reinforcing the need for rapid production and characterisation.

1.2.5 Antibody fragments

Recombinant DNA technology has provided the potential to define antibody affinity contributing significantly to their success in both analytical and medical applications.

Phage display libraries, in particular, have been successful in producing antibodies specific to almost any target (McCafferty et al., 1990). This technology uses probing of a library of 10^7-10^{10} protein coding combinations with varying diversity (Qin et al., 2007), for affinity to the chosen molecule and can be carried out using DNA recombination, human naïve or semi-synthetic libraries. Phage display can be used to develop novel binding fragments out of a library of around 10-100 fold the diversity of a naïve library. Thus screening of phage libraries can generate many potential candidates with antigen-binding properties (Hoogenboom and Chames, 2000).

Although the scFv products of phage display can be manufactured as whole antibodies, there are often advantages of using antibody-based derivative proteins instead. For instance initial manufacture and characterisation of proteins is more easily carried out in different formats including scFv-Fcs.

Antibody derivatives are formed of rearranged polypeptide chains to make novel antibody fragments such as single chain variable fragments (scFv) and diabodies amongst others (see Figure 1.4 Antibody Fragments). These retain the specific antigen binding properties of a whole antibody but may provide an improved bio-distribution *in vivo* (Holliger and Hudson, 2005). Whilst the large majority of regulatory submissions remain for whole antibodies, fragments are also entering clinical trials with increasing frequency (Nelson, 2010; Reichert, 2015), and as such antibody based therapies are expected to continue as one of the most profitable areas of the pharmaceutical industry in the next decade (Beck et al., 2010; Aggarwal, 2014), without necessarily remaining based on whole antibodies.

Fab fragments and single chain variable fragments (scFv) are the most common small antibody molecules used. Advantages of these smaller molecules include simple synthesis that can be achieved using bacteria and thus a reduced cost of production. The smaller size and weight of the fragments also changes the pharmacokinetics once in the body, essential when penetration of solid tumours is required. For a comprehensive review of single chain variable fragments and other antibody derivatives used as therapeutics see Gebauer and Skerra, 2015 or Holliger and Hudson, 2005.

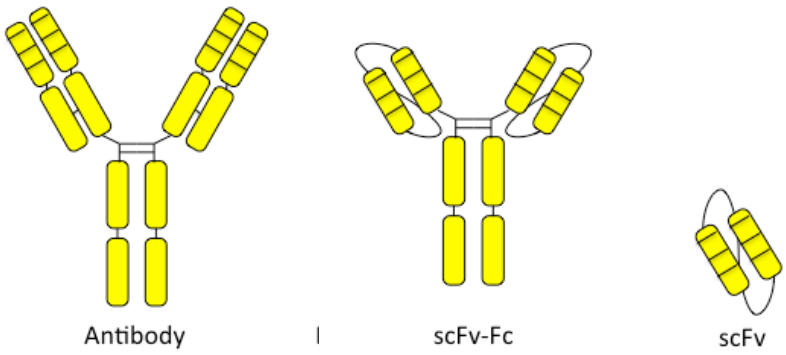


Figure 1.4 Antibody Fragments. Left to right – a whole IgG antibody, a single chain variable fragment-Fc fusion protein (scFv-Fc) and a single chain variable fragment (scFv). Complementarity determining regions are marked as in Figure 1.2.

Applications of antibody fragments include conjugation to toxins, radioactive labels and bioactive components such as enzymes. Using this set of conjugative payloads antibody fragments can be used to image, diagnose and treat disease with increased efficacy. However those antibody structures without an Fc region lose activation of FcyR or complement and thus immunomodulation of the cytotoxic cells of the innate immune system. Engineered multivalent antibody fragments can specifically bind to more than one antigen, with different target affinities at each variable region. There are many different formats of multivalent antibody fragments; some of which are designed to address the lack of an Fc using specificity to an antigen of choice and to innate immune cells. Multivalent antibody fragments can also be used to bring other cells and molecules into close proximity (Morrison, 2007).

Dual variable antibodies along with a range of multivalent antibodies have two or more antigen specificities on a single variable region achieved by insertion of a polypeptide loop into a CDR region or antibody variable domain linking. Advantages of this include increased avidity for antigen targets and improved cell targeting (Gu and Ghayur, 2012; Wu et al., 2007).

Conjugation of an scFv to an Fc fragment can reinstate immunomodulatory properties and provides a convenient cloning platform for scFv fragments produced via phage display libraries. However conjugation to an Fc also increases the size and complexity

of the molecule making mammalian expression necessary due its glycosylation requirements.

1.3 Bioprocessing

Bioprocessing of recombinant proteins is the procedure whereby a transgenic cellular expression system is grown under fermentation conditions to produce a protein and its subsequent recovery and purification. The process can be broadly split into upstream, fermentation and downstream stages. Upstream stages include protein engineering, expression plasmid design and transgene integration into host cells whilst fermentation is the growth of the host system and expression of the recombinant protein. Downstream processing is concerned with separation and recovery of the product through to polishing and formulation of the final product.

Expression systems used to produce proteins using recombinant DNA technology include simple microorganisms, mammalian cells, transgenic plants, insects cells and animals (Andersen and Krummen, 2002; Assenberg et al., 2013). Suspension-based microbial and mammalian cell culture is particularly well suited to large-scale fermentation that can be scaled up from laboratory research to meet industrial demands.

The cost of manufacture of recombinant proteins in mammalian cell expression systems is exceptionally high compared to those from microbial sources. However COG (Cost of Goods)/g is reducing thanks to improvements in upstream technology and feeding providing mammalian cell densities in excess of 1-1.5x10^7cells/mL and yields of 8g/L, (Farid, 2007; Hacker et al., 2009). The cost of mammalian cell culture, lengthy production times and complex down stream processing combine to increase costs for patients (Birch and Racher, 2006).

Addressing these issues is a priority for industry. Synthetic approaches to manipulating metabolic and biosynthetic pathways have already provided improvements in antibody production and alternative expression systems are also being explored (Wurm, 2004, Durocher and Butler, 2009). Downstream processing

and recovery techniques have also improved by utilising novel solid-liquid separation and chromatographic recovery steps (Warikoo et al., 2012).

1.4 Expression of recombinant proteins

Expression of recombinant proteins has for many years been carried out in a number of host systems including yeasts, bacteria, plants, transgenic animals and both cultured insect and mammalian cells. The advantages and disadvantages of the main expression systems are listed in Table 1.1 Expression system advantages and disadvantages. The decision on which expression system to use is often dictated by the eventual function of the protein and therefore it's necessary quality, production speed or scale and its cost.

Microbial expression systems are used to produce the majority of recombinant proteins at industrial and laboratory scale thanks to their simple and low cost requirements for growth. Strains of *Escherichia coli* and *Bacillus subtillis* are widely used bacterial expression systems (Demain and Vaishnav, 2009). They are very well characterised and many fully sequenced strains are available that can be easily manipulated using synthetic and molecular biology approaches. Yeast expression systems are also widely used, especially *Saccharomyces cerevisiae* and *Pichia pastoris*, as they are capable of exceptionally high yield and can perform some glycosylation on more complex proteins (Cregg et al., 2000; Miller et al., 2005; Mattanovich et al., 2012).

Microbes remain ideal expression systems as they are highly cost effective, allow for simple, high yield fermentation systems and grow to very high cell densities (Demain and Vaishnav, 2009). However not all systems are appropriate for the production of complex proteins such as antibodies and Fc conjugates due to their size and complex nature (Buck et al., 2013; Jacobs et al., 2008).

Transgenic expression system	Advantages	Limitations	Reference
Bacteria	Rapid expression, yield, simplicity, cost effective and well suited to mass production (scalable).	Disulphides hard to express, no glycosylation, endotoxins, inclusion bodies require refolding.	Terpe, 2006
Yeast	Yield, cost effective, high cell density, rapid growth, some glycosylation, stable strains, and good approval rate.	High mannose type glycosylation limits complex protein expression, very high cell density for DSP.	Mattanovich et al., 2012
Insect	PTM, proper folding, strong promoters, yield, approval rate, large proteins possible, scale up.	Different oligosaccharide chain extension, inactive cleavage sites, high O2 demand.	Cox, 2012
Mammalian	Large complex proteins, yield, scale up, convenience for approval.	Cost of media, time for stable cell selection, limited cell density, process implications (shear).	Matasci et al., 2008; Wurm, 2004; Zhu, 2011
Plant	Cost effective, scale out, PTM, low safety risk, some complex proteins produced, simple DSP.	Space and control of transgenic plants, limited PTM, immature technology.	Franconi et al., 2010; Pogue et al., 2010
Animals	Yield, processing in place (e.g. dairy), approval.	Cost, time and potential safety concerns	Wang et al., 2013

Table 1.1 Expression system advantages and disadvantages. Many cell types can be used for recombinant protein expression, however some are not currently applicable for production of therapeutic proteins for *in vivo* use.

1.4.1 Expression system glycosylation

Unlike translation of a polypeptide from mRNA, the glycoprofile of a protein is not DNA template driven. Considerable differences can be found in the glycoprofile of recombinant proteins, as glycosylation can be dependant on expression system and environment. This heterogeneity is termed either macroheterogeneity (i.e. whether a residue is/ is not glycosylated), or microheterogeneity (i.e. which oligosaccharide structure has been attached to a site) (Potgieter et al., 2009).

The necessity for correct protein glycosylation, especially in therapeutic use, can be demonstrated by the reduction in effector function or even immune reaction to some glycosylated proteins. This can range from non-functional proteins resulting from bacterial expression, where no glycosylation is present (Buck et al., 2013), to patient immune response to high mannose or xylose containing oligosaccharides from yeast and plants (Demain and Vaishnav, 2009; Jacobs et al., 2008; Jefferis, 2012). An example of the different host glycosylation structures are illustrated in **Figure 1.5 Examples of yeast, plant, insect and human glycosylation structures**.

Hence, although microbial expression systems are used to produce the majority of industrial recombinant proteins, those requiring glycosylation for correct structure and function, such as Fc containing proteins, are preferentially expressed in mammalian host systems (Wurm, 2004; Zhu, 2011; Lai et al., 2013; Dean and Reddy, 2013).

This challenge is being addressed using synthetic glycoengineering of expression systems in order to create human-like glycosylation structures as discussed by Durocher and Butler, 2009 and Potgieter et al., 2009 including successes such as Novozymes (Denmark) recombinant human albumin *S. cerevisiae* expression system. Further glycoengineering of recombinant proteins has been demonstrated by altering antibody effector function after reduction of fucosylation present on the CH2 domain of the Fc region of IgGs for improved ADCC (Okazaki et al., 2004; Shields et al., 2002).

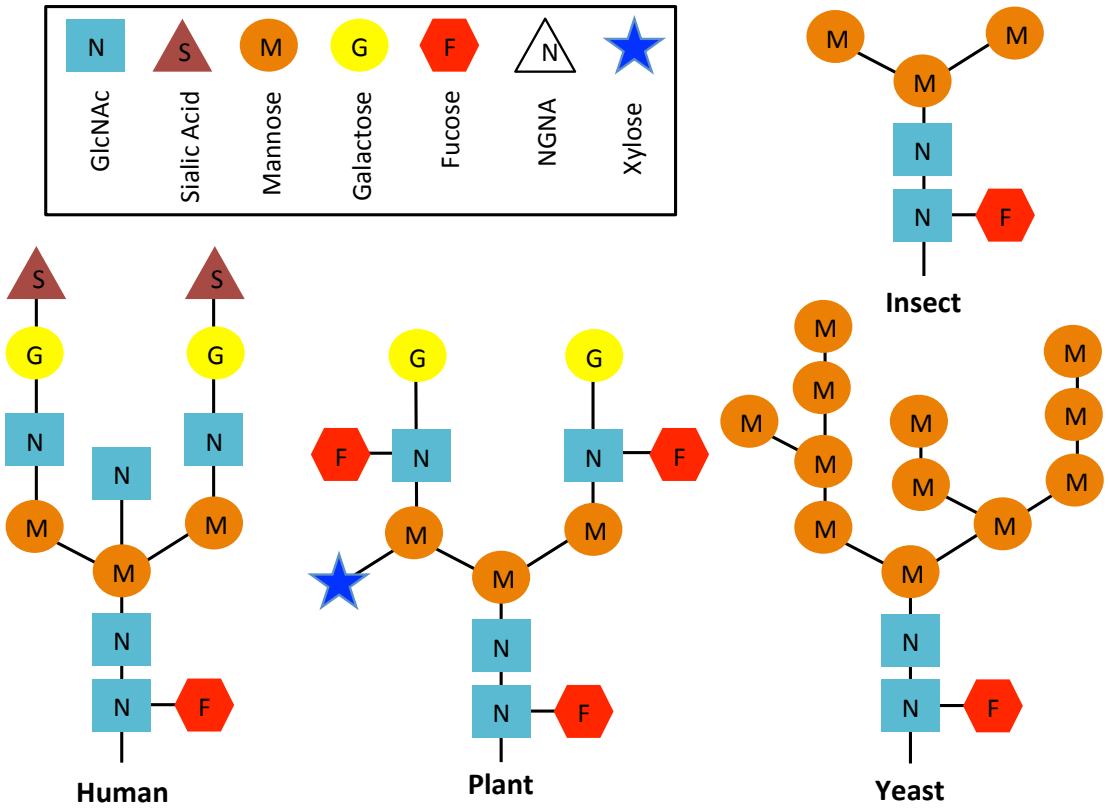


Figure 1.5 Examples of yeast, plant, insect and human glycosylation structures. Adapted from Jefferis, 2008 and Jacobs and Callewaert, 2009. Glycosylation structures vary substantially between species types. These differences can have significant impacts on immunogenicity and therefore host system of choice during protein expression**.** NGNA- N-Glycolylneuraminic acid.

1.5 Mammalian cells as expression hosts

Mammalian cells are used to produce the majority of biopharmaceuticals. The popularity of mammalian expression systems stems from the relative ease of product approval, complex posttranslational glycoprotein modifications and a large base of methodology to support their use (Wurm, 2004; Zhu, 2011; Lai et al., 2013). The average amount of recombinant protein produced in mammalian cells has increased 100 fold since the 1980s (Wurm, 2004). These increases in productivity are owed to several factors including the generation and selection of cell lines with high viability, improvements in fermentation techniques and improved expression plasmid design.

Mammalian expression systems that meet today's industrial scale protein demands became a reality after adherent cells were adapted to growth in a suspended state. Adherent cells are inefficient for mass cell culture, as they require high surface areas and maintenance. Microcarrier based cell culture expands the available surface area for cell cultivation of anchorage dependant cells using small particle spheres of around 500 μm diameter (Justice et al., 2009). However adaptation of anchorage dependant cell lines to growth in suspension can be achieved making the use of micro carriers redundant, saving costs and simplifying downstream processing of material at industrial scale.

Cells rely on signals and nutrients provided in serum for proper growth. In mammalian cell culture foetal bovine serum (FBS) is used as a substitute for the natural serum that surrounds a cell. However serum from bovine sources has potential for viral contamination and remains expensive therefore is unsuitable for large recombinant therapeutic production. Cells adapted to growth in serum free media remove the problems associated with animal based products for therapeutic use. Sequential adaption of cells can be achieved by gradually swapping serum-based media for serum free media over a period of passages (Sinacore et al., 2000).

Suspension adapted mammalian cells are grown in shaken flasks at laboratory scale and fermentations can now be carried out at 20,000L capacity in stirred tank bioreactors for industrial production of recombinant proteins. There are multiple platforms for mammalian cell culture aiming to increase productivity and cost

efficiency whilst reducing reactor downtime e.g. disposable reactors such as the Wave bioreactor (GE Healthcare) or continuous perfusion feeding strategies.

1.5.1 Common mammalian expression hosts

A number of mammalian cells are in common use for protein expression taking advantage their unique properties. Cells from human, Chinese Hamster, monkey and murine donors are used regularly (See Table 1.2). However CHO cells remain the dominant expression system, currently used to express over 70% of recombinant therapeutics (F. Li et al., 2010). There are a number of reasons for the popularity of CHO cells including their yield, high cell density, regulatory familiarity and established production protocols (Durocher and Butler, 2009; Kunert and Reinhart, 2016). Although not human cells they can achieve glycosylation more similar to humans than murine cells (see Figure 1.6)

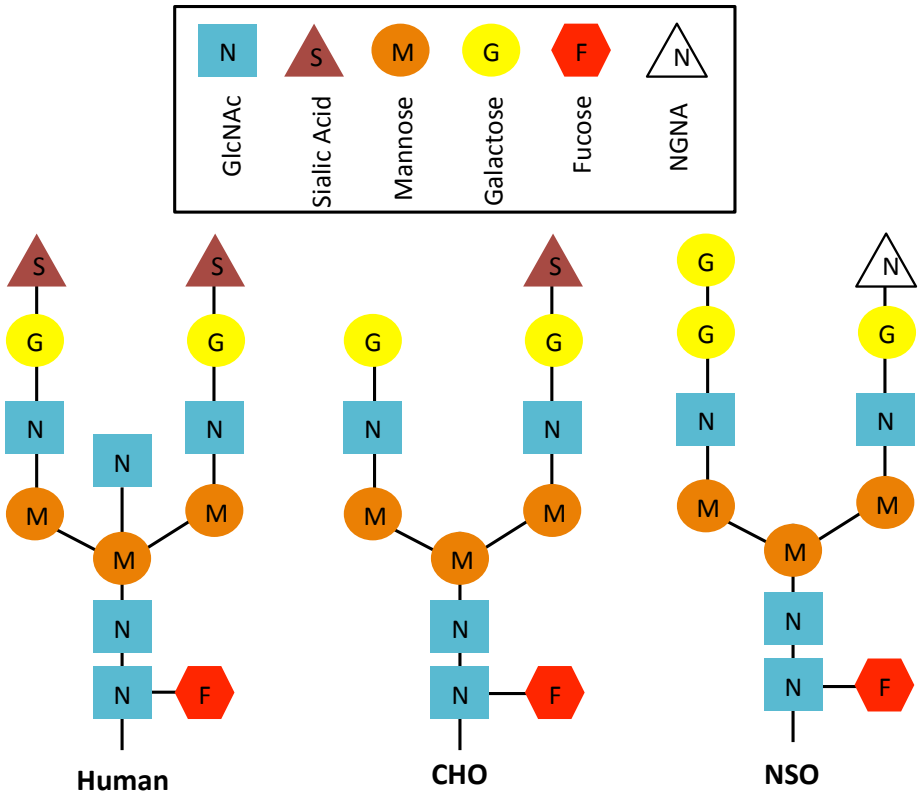


Figure 1.6 Comparison on human, CHO and NSO glycosylation (Adapted from Fliedl et al., 2014 and Butler, 2005). Glycosylation structures remain relatively constant across recombinant protein production mammalian host systems.

Expression host	References
Chinese Hamster Ovary (CHO)	Clincke et al., 2013; Kim et al., 2013; Wernicke and Will, 1992; Ye et al., 2009
Human Embryonic Kidney (HEK293)	Backliwal et al., 2008a; Cervera et al., 2011; Durocher et al., 2002; Thomas and Smart, 2005
Murine Myeloma (NS0, SP2/0)	Dietmair et al., 2012; Wurm, 2004
Human Embryonic Retinoblast (PERC.6)	Lewis et al., 2006; Pau et al., 2001; Zhu, 2011
African Monkey Kidney (COS, VERO)	Fliedl and Kaisermayer, 2011

Table 1.2 Mammalian expression hosts. Multiple expression host system have been generated from mammalian cells of different species however CHO cells remain the standard industrial cell line for recombinant protein expression. Further descriptions of cell lines can be found in Khan, 2013.

1.5.2 Expression plasmid design

Non-viral vectors are most often used for the expression of recombinant therapeutic proteins over retroviral vectors. Expression of recombinant proteins in mammalian cells is generally driven using strong, constitutive promoters. Popular viral promoters include the cytomegalovirus (CMV), human T-cell leukaemia virus (HTLV) or the simian virus 40 (SV40). These viral promoters can be combined with cellular equivalents such as constituents of the elongation factor 1 alpha (E1Fα). Composites of viral and cellular promoters are often used to drive particularly strong expression often combined with elements designed to effect mRNA transcription and half-life.

Transcriptional elements involved in mRNA processing involve the inclusion introns that have been shown to play significant role in the increased protein expression during (pre) mRNA splicing (Le Hir et al., 2003). Translation initiation can be improved using elements such as the Kozak sequence, a consensus sequence that is present at the region immediately before translation. This has been shown to differ according to expression system (Nakagawa et al., 2008).

The posttranslational modifications (PTM) and fate of proteins can also be modified in the design of plasmids. These include the addition of polyadenylation and secretion signal sequences that control the location of recombinant proteins. Further protein engineering at this stage includes the addition of affinity tags and selection markers as discussed in 1.7.4 Affinity tags and 1.6.5 Stable transgene integration.

1.5.3 Transfection

Recombinant protein expression in mammalian cells is achieved by the introduction of transgenic DNA. There are a number of techniques available that move DNA into the nucleus including mediation by transduction (viral delivery) or transfection (chemical or physical methods) (see Figure 1.7). Introducing foreign nucleic acids into mammalian cells can be used for analysis of gene and protein function, knocking out genetic elements as well as recombinant protein production.

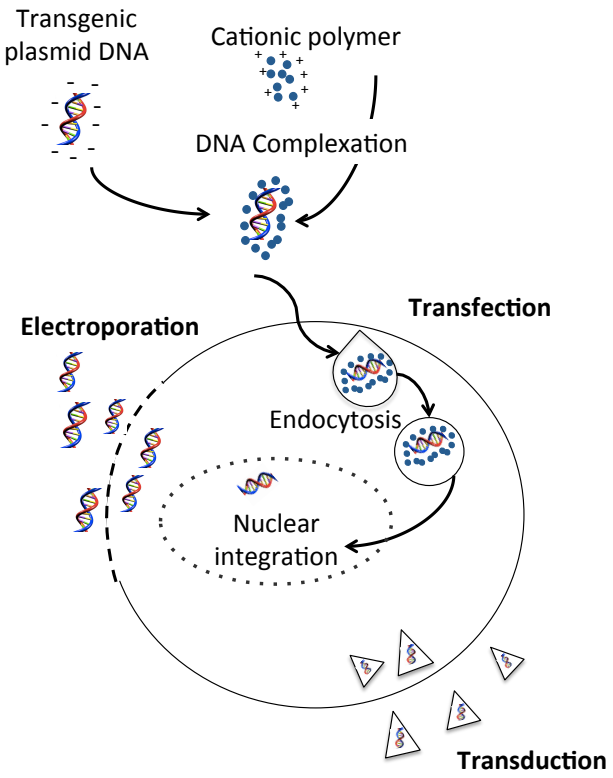


Figure 1.7 Introducing DNA into mammalian cells. Examples of three methods for DNA integration into mammalian cells including electroporation induced pore formation, viral mediated transduction and cationic liposome mediated transfection. All three methods can be used for transient or stable cell generation, however this diagram is far from exhaustive.

Whilst transduction is an excellent gene delivery system, it is limited by its nucleic acid payload capacity and potential safety concerns in therapeutic use. In contrast transfection via chemical methods, such as cationic liposome formation, are the most widely used, as they are simple and effective. The range of available transfection reagents can also be expensive and in many cases have cytotoxic effects. Physical methods often rely on transient membrane pore formation e.g. electroporation (Ehrhardt et al., 2006), however these techniques can be reliant on expensive equipment. A full discussion of the advantages and disadvantages of gene delivery methods can be found in Kim and Eberwine, 2010.

The exact methods of nucleic acid entry and translocation into cells is not yet fully characterised with conflicting evidence in the literature (Akinc et al., 2005;

Benjaminsen et al., 2013). However it has been shown that cell passage through mitosis is a prerequisite for transfection (Mortimer et al., 1999) using popular cationic polymers such as polyethylenimine (Durocher et al., 2002; Ehrhardt et al., 2006). Hence cell cultures under going rapid division are necessary for successful transfections of recombinant protein expression systems. Translocation of DNA to the nucleus or episomal space for transcription and translation is necessary for protein expression (Jäger et al., 2013).

1.5.4 Transient transgene expression

Transient gene expression (TGE) is widely used for cell research (Elias et al., 2003) and rapid production of recombinant proteins in mammalian cells (Wurm, 2004). Advantages of TGE include simple methodology and speed of expression; purified recombinant protein can be obtained within a few days, whilst stable gene expression (SGE) requires a laborious, time consuming selection process often taking weeks to months (Backliwal et al., 2008c). Transient gene expression therefore lends itself particularly well to early stage expression of proteins such as biotherapeutic for preclinical characterisation and low quantity assays.

Transgenes that are not integrated into the genome are removed from the cell within a matter of days or diluted during cell division (Matasci et al., 2008). As well as providing valuable material for preclinical performance studies, TGE has also been utilised in the analyses of manufacturability of candidates.

Transient gene expression can be used to predict the expression characteristics of recombinant proteins in given expression systems expression characteristics of similar recombinant proteins are not necessarily the same (Bentley et al., 1998; Pybus et al., 2014b). Ye et al., 2009 showed that there was no difference in glycosylation profile between transient and stabley expressed proteins therefore TGE is often used to produce the first quantities of novel proteins for use preclinical and early *in vivo* experimental work prior to committing to stable cell selection (Agrawal and Bal, 2012).

The high transfection efficiency and yield of the human embryonic kidney (HEK293) cell line has meant it has been widely adopted over CHO cells (Sun et al., 2008). However as the majority of industrial scale culture uses CHO cells in stable form there is also a demand for high expressing transient systems in CHO to improve modelling of protein activity from stable cells (Derouazi et al., 2004). Although ideal for laboratory scale expression, transient transfection has limited usage in large-scale production due to difficulties in producing consistently homogenous products, requirements for large amounts of DNA (>mgs) and repeated transfections to continue production.

The lack of selection requirements simplifies vector construction for transient systems however expression performance can be improved via design of transgene vectors to increase the expression of the DNA. Examples include incorporation of transcription factor X-Box binding protein 1 (XBP1) to improve secretion (Codamo et al., 2011), the cytomegalovirus (CMV) promoter that is commonly used (Boscolo et al., 2011; Zhu, 2011) and by optimisation of transfection cell densities and methods (Backliwal et al., 2008b; Durocher et al., 2002). Using these systems transient protein expression can produce up to 1g/L quantities of protein in HEK293s (Backliwal et al., 2008a).

1.5.5 Stable transgene integration

Transgenic DNA integrated into the genome via homologous recombination provides long term, defined expression of a transgene in a cell. Once a transgene is integrated into the genome it is expressed constituently as part of the cells proteome removing the need for further transfections. The advantages of stable cell integration include constitutive expression, high yields and genomic heritage of daughter cells enabling high cell density fermentation and cryopreservation of cell lines.

Stable genomic integration of transgenes is a rare event; hence cells are selected using a marker that confers a selection advantage on those cells. As such transgene constructs destined for stable cell generation contain markers, antibiotic resistance genes or metabolic enzymes, included in the expression vector. Cell selection using antibiotic resistance markers employs growth in selective media to kill those cells without the corresponding resistance gene, similar to bacterial selection. Problems

with antibiotic selection occur when multiple resistance vectors are used with a single cell line (i.e. co-transfection) and the need to include unnecessary genetic elements in the cell.

Alternative selection methods used in CHO cells utilise knockout cell strains that are deficient for enzymes involved in key metabolic reactions such as nucleotide synthesis (dihydrofolate reductase (DHFR)) or glutamine synthesis (glutamine synthetase (GS)). In both instances inclusion of DNA coding for the missing metabolic enzyme in the expression vector confers a selection advantage on stably integrated cells. Metabolic selection involves culturing transfected cells in a media negative for the enzyme product i.e. glutamine or the nucleotide pre-requisites hypoxanthine and thymidine (Wernicke and Will, 1992). For an in depth description of selection markers see (F. Li et al., 2010).

A comparison of transient and stable gene expression can be found in Table 1.3 Transient and stable gene expression.

Table 1.3 Transient and stable gene expression

	Time period	Expression system	Yield	Scale	Application
Transient Expression	48hrs – 14 days	HEK293 and CHO	10mg-100mg/L	<100L	Preclinical and research
Stable Expression	3 Months+	CHO	g/L	Up to 20,000L	Large scale for therapeutic use

1.5.6 Mammalian cell fermentation

Shake flask and bioreactor growth of suspension-adapted cells enables very high cell density cultures at large and small-scale operation. Key factors in the ability to gain

high cell density include the design and optimisation of media and feeding strategies, environmental control in bioreactors and cell engineering approaches.

Media composition for suspension cell culture purposes uses chemically defined media for most batch production processes. This contains a mixture of glucose, specific lipids, vitamins, inorganic salts, amino acids, growth factors and trace elements without the need for animal sourced products. Additionally concentrated feed can be used to replenish spent media components in fed-batch approaches that extend cell culture periods and therefore increase productivity (Lu et al., 2013; Sauer et al., 2000). The optimisation of feeding strategies and constituents can be carried out using a multifactorial design of experiments approach. Semi-continuous cultures using perfusion systems that replace media whilst retaining cells can be used to increase culture period and viable densities significantly however perfusion reactors remain costly compared to current fed-batch processes (Pollock et al., 2013).

Stirred tank bioreactors are the most commonly used mammalian cell culture platform however the need for reactor sterilisation and cleaning of these reactors adds down time and requires validation or cGMP processes. A range of single-use reactors are now in use that can be used with most suspension cultures including roller bottles the WAVE bag (Singh, 1999), pneumatic bioreactors (Kim et al., 2013) and singe-use stirred tanks (Odeleye et al., 2013).

Cell engineering strategies can be employed to attain improved viability, and therefore productivity through tolerance to stresses such as shear, osmotic pressure and toxic accumulation. Inhibition of apoptotic pathways can be achieved by engineering strains to over express *bcl-2* or inhibition of caspase 9 and 3 by addition of active site blocking peptides or bacilovirus p35 insertion (Vives et al., 2003). Alternatively chemical inhibition of caspase 9 and 3 has been shown to increase cell viability by up to 33% and improve antibody production titre by 20% in CHO cells (Arden et al., 2007). Metabolic engineering such as lactate dehydrogenase (LDH) deficient strains can produce as high as a 50% reduction in lactate synthesis and a cell density increase of 30% (Chen et al., 2001).

These methods have successfully increased culture viability and enhanced resistance to stresses such as toxic accumulation, nutrient and oxygen limitation or shear. However the overall protein production from these genetically modified (GM) cell lines is not as great as was anticipated A combination approach to improving cell viability has been suggested that could incorporate multiple modifications (Dinnis and James 2005; Dixit et al., 2003).

1.5.7 Measurements of protein expression

Fermentation and feeding strategies are designed to balance the need to promote cell growth and maintain volumetric productivity of a cell culture (Dean and Reddy, 2013). Calculating specific parameters, such as the specific and volumetric productivity, allows the comparison of protein expression cells on a like for like basis. Recombinant protein yield is dependent on individual production rate of cells and the number of viable cells in culture; the function of these elements is the volumetric productivity see Equation 1 Volumetric productivity

U x *q*P= volumetric productivity

Equation 1 Volumetric productivity

U= cell density, *q*P= single cell (specific) productivity.

Equation 2 Specific productivity of a cell culture in picograms per cell per day (pg/cell/day), also known as single cell productivity;

$$q_{mAb} = \frac{m_{mAb}}{ICA}$$

Equation 2 Specific productivity

^{q}mAb = Specific productivity, ^{M}mAb = Mass of antibody in supernatant, *ICA* = Integral cell area

Where m_{mAb} is the total mass of antibody in the cell supernatant and (ICA) integral cell area is calculated as described in Equation 3 ICA (Integral Cell Area);

$$ICA = \frac{(N - N_0) \times t}{log_e (\frac{N}{N_0})}$$

Equation 3 ICA (Integral Cell Area)

ICA = Integral Cell Area, N = number of cells at time point 2, N_0 = No of cells at time point 1, *t* = Time.

Where *N* and N_0 are the number of viable cells at the end and start of the culture period and *t* is culture time.

Maintaining a high specific yield along with a high viable cell count over an extended time period delivers optimum productivity for industrial cell culture.

However the volumetric productivity of a cell culture can also be significantly affected by a number of factors such as cell cycle, temperature and nutrition. Therefore these factors must be tightly controlled. Glucose is the main source of energy for cells yielding lactate as a waste product that, if accumulated, can cause a reduction in cell growth and protein productivity (Eibl et al., 2009). Glutamine metabolism is also a key factor in cell growth contributing up to 30% of cell energy via TCA cycle oxidation with the excretion of ammonia as a waste product. Ammonia is particularly important as high concentrations (>5mM) can affect glycosylation of proteins (Xie and Zhou, 2005). Measuring the consumption of glucose and excretion of lactate allows for appropriate cell feeding strategies to be designed based on the state of cell metabolism.

The effect of temperature on recombinant protein expression and growth of cells can be significant. Strategies for enhanced protein production include a temperature reduction to induce mild hypothermic conditions resulting in an increased portion of cells in G1 phase and a significant increase in protein yield compared to cultures kept at 37C. The glycosylation profiles of proteins from cell cultures can also be affected. (Agrawal and Bal, 2012; Sunley and Butler, 2010)

In large-scale mammalian bioprocessing of antibodies a significant amount of glyco-heterogeneity is found due to affects of environmental factors on glycosylation e.g.

temp, ph (Zhu, 2011). Glycosylation of antibodies produced at large scale is therefore an area of intense research with the aim of producing a uniform product across multiple batches of antibody using environmental and genetic manipulation (Hossler et al., 2009).

1.5.8 Improving protein expression

Recombinant protein expression can be improved by tackling the challenges of unequal genome expression, gene silencing and transcriptional control. The genome is not expressed equally (e.g. heterochromatin and euchromatin) thus DNA integrated at areas of high expression will be translated at a greater rate, this is the position effect (Feng et al., 2001). To gain increased volumetric productivity from a culture a clone with high transgene expression can be selected and cultured however this process can be time consuming.

Site-specific integration is a method of targeting the locus of transgene integration to a transcriptionally active site allowing rapid generation of stable cells with high expression of a transgene. These genomic 'hotspots' can be targeted using recombinase specific DNA integration sites that are transfected into the genome and assayed for high expression. Subsequent co-transfection of a vector containing the corresponding recombinase and the recombinant DNA of interest is then integrated via site-specific recombination (Liu et al., 2005).

As the sites of integration are genetically identical in the cell population, transfection results in an isogenic population that can be easily screened using selectable markers. Site-specific integration removes many of the key challenges in screening for high-expressing stable cells.

Alternatively incorporation of specific promoters and epigenetic modifications into a transgenic vector can ensure high expression at any site of integration. The universal chromatin-opening element is a region of DNA that has a set of promoters usually used for housekeeping genes allowing constitutive high expression of recombinant proteins. Heterochromatin formation can reduce or silence transcription of a gene. Non-methylated CpG vectors can be used to successfully reduce heterochromatin

formation around an integration site allowing expression of transgenic DNA without the risk of gene silencing to improve stable cell expression (Boscolo et al., 2011).

Expression ratios of some constructs such as antibodies rely on co-transfection of multiple plasmids in many cases as antibody heavy and light chains are expressed at different rates from the genome. The optimum ratio of transgene insertion is therefore not an equal 1:1 heavy chain (Hc) – light chain (Lc) ratio. It has been shown previously that whilst heavy chain expression is a limiting factor in productivity, light chain expression is expressed at 2.25-fold that rate. However during folding and secretion of antibodies, light chain availability becomes the limiting factor. The highest expression and secretion rates have been found at a Hc:Lc ratio of 3:2 in CHO cells (Schlatter et al., 2005).

1.6 Downstream processing and primary recovery of recombinant proteins for therapeutic application

Recombinant proteins, as discussed in section 1.1, have a wide range of applications from therapeutic use to food processing and bioremediation. As such the techniques involved in purification and storage of proteins varies as much as the products themselves ranging from simple protein recovery through to cGMP-compliant processes. Proteins destined for therapeutic use have the most stringent controls on downstream processing protocols requiring validation to the highest levels ensuring product quality and sterility.

In the past a substantial percentage of the high costs of mammalian derived proteins has come from fermentation however, recently production costs have become more heavily weighted toward downstream processing (González *et al*., 2003). The same advances that have improved cell growth and protein expression have therefore in turn created new challenges for downstream processing, leading to a focus on developing high capacity methods for the primary capture of protein from high cell density feeds.

Bioreactor based fermentation of expression hosts generates a liquid feed stream of varying viscosity dependent on expression host. The industrialisation of recombinant

protein production has meant increased bioreactor capacity with feed streams in excess of 20,000L now routinely processed.

Some feeds such as mammalian cells require a carefully considered approach to downstream processing, as they are more susceptible to cell disruption when compared to other host systems such as yeast and bacterial cells. Disrupted cells can introduce increased levels of feed contaminants such as DNA and proteases that effect protein quantity and further downstream processes.

1.6.1 General outline of downstream process unit operations

Process scale, expression system of choice and recombinant protein structure all affect the decisions made in downstream processing of these high value molecules however there are general unit operations are performed in most processing cases. Generally feed streams consist of both solid and liquid components that must be separated prior to protein capture via affinity, ion exchange or size-exclusion chromatography. After elution of the target protein a series of downstream processes are applied to further purify and reformulate the protein into its final state. Here the initial harvest and primary recovery strategies for a mammalian cell feed stream are discussed up to the point of primary product capture (Figure 1.8 General mammalian cell process flow diagram. **A simplified process flow diagram for expression and capture of a recombinant protein from mammalian cells including upstream expression system engineering, fermentation and DSP through to clean up (polishing) and formulation.**

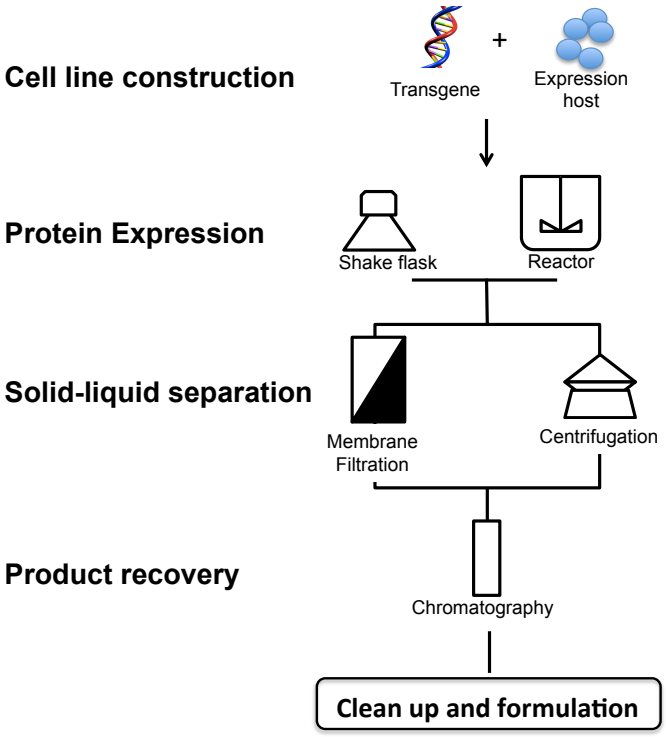


Figure 1.8 General mammalian cell process flow diagram. A simplified process flow diagram for expression and capture of a recombinant protein from mammalian cells including upstream expression system engineering, fermentation and DSP through to clean up (polishing) and formulation.

1.6.2 Solid-liquid separation

Recombinant proteins are usually secreted by mammalian cells into growth media via one of many available secretion tags (Kober et al., 2013) included in protein engineering stages. Solids typically make up around 40-50% of the feed in high cell density fermentation (Marichal-Gallardo and Álvarez, 2012), hence the first stage in product recovery is the segregation of solid and liquid elements. Primary processing of high cell density feed streams requires particular consideration to avoid cell stresses and shearing that could result in protein aggregation and cell lysis (Hutchinson et al., 2006; Kiese et al., 2008). The latter affecting the quantity and quality of product by host cell proteins (HCP) and host DNA contamination (Sandberg et al., 2006).

Typically solid-liquid separation consists of centrifugation and depth/micro filtration steps to remove cells and cell debris from feed streams (Marichal-Gallardo and Álvarez, 2012).

1.6.2.1 Centrifugation

At industrial scale batch processing reduces efficiency therefore a semi-continuous sedimentation approaches are widely used. Tubular bowl centrifuges accumulate solids against the wall of a rotating bowl whilst enabling liquid discharge in a continuous manner through the end. Although the technology is effective the disc stack centrifuge thanks to its partial discharge function allowing continuous operation as well as a reduced shear effect on the feed stream has largely superseded it. The disc stack centrifuge consists of a set of plates arranged in a conical fashion, as feed enters liquids pass through the plates whilst solids accumulate on the underside and exterior of the bowl (for diagram see Marichal-Gallardo and Álvarez, 2012). As with the tubular bowl the liquid portion of the feed is discharged through the top of the centrifuge whilst the solids are separated to the underside of the plates. However disk stacks enable removal of these solids during operation in either partial or full discharge modes via opening of the bowl sleeve, enabling a continuous flow during the process.

1.6.2.2 Filtration

In order to prevent fouling of high expense chromatography resins, feed should be fully clarified prior to column application. Microfiltration has historically been used in biologics recovery as it enables separation of recombinant protein from both large and small remaining after centrifugation. Filtration can occur either via dead-end or tangential flow. Microfiltration units operate via exclusion of particles to form a caking layer on the surface of the membrane. However fouling via pore blockage or cake formation significantly affects membrane flux, requiring filter cleaning and replacement at regular intervals.

Depth filters, unlike membrane filters, utilise a porous medium that is able to retain particles throughout its matrix not just its surface. Depth filters for bioprocess operations generally come in disposable units and are constructed of diatomaceous

earth (DE), perlite and cellulose fibres. Filtration based on particle exclusion and internal adhesion enables removal of different size particles and can be combined with a more traditional membrane filter as the bottommost layer to ensure clarification. The combination of centrifugation and depth-filtration is now the industry standard for processing of mammalian-expressed proteins prior to chromatography (Marichal-Gallardo and Álvarez, 2012). Both depth and microfilters are available in single-use cassette systems that avoid validation challenges and make process integration simple.

Factors affecting the clarity of feed streams from these processes include the feeds turbidity and its flow rate. Both of these factors can be significantly affected by cell lyses during bioreactor fermentation, centrifugation or filtration. The shear affects experienced by cells have been shown previously to impact the protein yield and filter area required or feeds in scale down models (Hutchinson et al., 2006).

1.6.3 Affinity tags

Recombinant protein purification relies on affinity chromatography for primary recovery in most cases. In terms of antibody purification, protein A from *S. aureus* has affinity for the junction between the CH2 and CH3 domains (Figure 1.2 Antibody IgG1 Structure Protein A based chromatography is used in the purification of two thirds of antibodies (Vunnum et al., 2009). However products without natural affinities are less easily purified, hence recombinant protein purification has long relied on utilisation of engineered affinity tags which can be used to facilitate capture in addition to improving expression and solubility (Young et al 2012). A large selection of affinity tags are in use as described in (Arnau et al., 2011). The polyhistidine repeat tag however, remains the major workhorse for affinity tag purification due to the simplicity of N or C terminal fusion and purification via IMAC (Terpe, 2003). Protein capture via a polyhistidine repeat tag is often achieved using repeated histidine residues at the C-terminal region of proteins, most commonly with a 6 residue repeat, although the number of histidine residues and tags (i.e. dual polyhistidine tags) can vary.

1.6.4 Chromatography and primary protein capture

Primary recovery strategies are highly dependent on the characteristics of the protein and cell type used to express them.

Affinity chromatography makes use of highly specific, reversible interactions between molecules to selectively retain mobile proteins on an immobilised ligand. Once a target protein has been captured altering conditions within the column reduces affinity and the product is eluted. These interactions can be based on native protein properties such as antibody Fc ot via the addition of purification tags engineered into a protein (e.g. histidine repeat tag) that allows protein capture based on immobilised metal affinity chromatography (IMAC) (see section 1.7.4).

Purification ligands are immobilised on a stable material such as Sepharose to form a chromatography resin, usually in bead form that is then packed into a static bed. The height and density parameters of this bed are adjusted dependant on scale of purification. Traditionally chromatography beads are formed into solid phase beds averaging around 80-100μm in diameter. A feed stream that is applied to a packed bed column cannot contain a large number of particles due to blocking and eventual cake build up on the bed.

Expanded bed adsorption (EBA) resins are packed at a reduced density sometimes referred to as a fluidized bed. Bead sizes in EBA vary but are often between 200-1000μm. Unlike packed bed chromatography, mixtures of particulate matter and target protein can be applied to EBA resins due to increases in pore sizes and reduced density of packing.

1.6.5 Advances in protein recovery

As upstream processing technology has improved the associated cost has come down whilst the cost of DSP has remained relatively constant. Overall, the cost considerations have become more weighted towards downstream processing as protein titres and cell densities increase (González et al., 2003). This has affected the demands of primary recovery and alternative primary recovery techniques are now

being harnessed including both resin and column designs that require less stringent clarification of media prior to column passage.

Large bead diameter chromatography resin utilises stationary phase agarose beads typically between 300-500μm in diameter creating a semi-porous bed that allows particles to flow through it.

Unlike typical axial flow systems, radial flow chromatography (RFC) consists of two concentric cylindrical porous frits holding a stationary phase between them. In RFC feed flows from the outer to the inner surface across the radius of the column providing a minimal pressure drop, improved elution peak resolution due to trapezoidal column geometry and greater operational flow rates (Besselink et al., 2013)(Fig 2.1, Table 1.4). RFC columns can be packed with many resins depending on frit design, including ion exchange (IEX), immobilized metal affinity chromatography (IMAC) or protein A/G.

Table 1.4 Pros and Cons of Radial and Axial flow based chromatography systems.

Radial Flow	**Axial Flow**
Scalability - bed height does not change with increased scale, rather the column is expanded in length.	Industry standard – Axial flow is the industrial standard for chromatography therefore is well optimized and efficient.
Pressure drop – Radial flow columns have a reduced pressure drop	High capture rates – Simple to run sequentially to obtain improved capture.
Concentration - radial concentric shape of the solid phase within the column facilitates a concentration effect of the product upon elution.	Scale down– Easy to reduce in size for small scale purifications.
Flow rate – due to the reduced pressure drop the flow rate can be increased compared to axial columns.	
Flexible - Both column types can be used with multiple different types of resins including bead type (affinity, Ionic exchange etc) or solid phase type (Expanded bed, fluidized bed or large bead).	

1.7 Motivation and rationale

This thesis aims to develop and compare methods for simple, cost effective and rapid expression and recovery of recombinant proteins for early screening of novel drugs. Transient and stable gene expression in mammalian systems was carried out using a novel scFv-Fc and its clinically relevant target, CEA, as model proteins. A process for primary capture of protein from unclarified mammalian cell feed streams was developed and characterized allowing protein recovery directly from bioreactors during harvest. Together these techniques aim to dramatically reduce the cost and time associated with production, purification and screening of recombinant protein therapeutics, including both the antigen target and binding antibody.

Firstly, the anti-CEA scFv-Fc was expressed via transient transfection of HEK293F cells and stable expression in CHO cell pools. The resulting yield and protein quality was compared between the two systems.

Secondly, a process was developed using a scale down radial flow chromatography system packed with large bead diameter resin to obtain an integrated primary capture step with IMAC for unclarified mammalian cell fermentation feed streams. This removed the need for centrifugation and filtration of feeds prior to primary capture. The results form the basis of an interfaced primary recovery step applicable to the recovery of many recombinant proteins from unclarified mammalian cell feed streams.

Chapter Aims:

Aim 1) Develop a mammalian system for rapid, scalable and cost effective production of recombinant proteins for use in laboratory scale production.

Aim 2) Investigate methods to generate stable cell pools for high expression of a model protein and to compare scFv-Fc produced in stable and transient systems

Aim 3) Develop a process for protein recovery directly from unclarified mammalian cell feed streams and demonstrate reactor harvest integration.

Chapter 2 Materials and Methods

2.1 Cell lines

2.1.1 Cell line storage and recovery

Unless otherwise stated all cell lines were stored in liquid nitrogen at a density of $1x10^7$ cells/mL in standard media, as defined below, supplemented with 10% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Gillingham, UK). Suspension adapted cell lines were thawed from a single vial in a 37 °C water bath before addition to directly to 29mL of pre-warmed media in a 125ml Erlenmeyer vented conical flask with a 0.22μm filter from Corning Limited (Union City, CA) and placed in incubation conditions. Adherent cell lines were also thawed in a 37 °C water bath but were added to 5 mL of pre-warmed media and centrifuged at 500x*g* and re-suspended in fresh media before seeding tissue culture plates as described below and placing in incubation conditions.

2.1.2 Human embryonic kidney-293F

The HEK293F cell line was purchased from Life Technologies, Paisley, UK. Cells were maintained in Freestyle serum-free media also from Life Technologies (12338-018) at a density of $0.5x10^6$ in 125ml Erlenmeyer vented conical flasks with a 0.22μm filter from Corning. Cell culture conditions were 37°c, 125 RPM and 5% CO_2 for all suspension shake flask cultures in an Stuit Orbital SJL1 incubator using a shaken platform from RS Biotech R+ (Ayrshire, UK).

2.1.3 Hela

Hela cells were obtained from Dr Darren Nesbeth at UCL Biochemical Engineering (University College London, UK). Cells were maintained in 5 mL of DMEM supplemented as above in T25 tissue culture flasks (Corning). H2Z-Hela cells, stably expressing flp recombinase targeting sites (FRT), were also obtained from Dr Darren Nesbeth and were maintained as above with the addition of 150 μg/mL zeocin (Life Technologies), (Cat. Num R250-01) to maintain selection pressure.

2.1.4 CHO-S and stably transfected CHO-S cells

Suspension and serum free adapted Chinese Hamster Ovary cells (CHO-S) (Life Technologies, Paisley, UK) or stably transfected CHO-S cells were seeded at a density of 0.3 million viable cells (MVC)/mL in 300mL of a 1:1 (v/v) mix of CD-CHO (Life Technologies, Paisley, UK) and EX-CELL CD-CHO 3 (Sigma Aldrich, St. Louis, MO) in 125mL Erlenmeyer flasks (Corning Limited, Union City, CA) with 0.22μm vented caps to a maximum cell density of $3x10^6$ cells/mL unless otherwise specified. All incubation conditions were 125rpm, 8% CO_2 and 37°C. A master and working cell bank was stored at low passage number (p2/p3) in liquid nitrogen and all cell stocks were maintained below $3\text{-}4x10^6$ cells/mL during regular passage.

2.1.5 Cell line maintenance

2.1.5.1 Adherent cells

Media was aspirated from adherent cell cultures and an equal amount of pre-warmed PBS solution was added and aspirated to remove residual serum. Pre-warmed 0.25% Trypsin solution (Sigma Aldrich) was added sufficient to cover the surface of the flask. The flask was returned to incubation conditions for 5 minutes before cells were dislodged by pipetting and re-suspended in fresh supplemented media for counting using a haemocytometer and a Motic AE 2000 (Wetzlar, Germany) microscope before subsequent dilution and plate seeding.

2.1.5.2 Suspensions cells

Cells in suspension were removed from culture flasks (30μL) by pipette and mixed with Trypan Blue stain (Sigma Aldrich) at an appropriate ratio dependent on cell concentration. Cell density and viability were determined by visualisation using a microscope with 10 μL of Trypan Blue and cell culture solution loaded onto a haemocytometer. Cell suspensions were diluted in the appropriate amount of pre-warmed media as calculated via the equation C1V1=C2V2. Where C1 is the current concentration, C2 is the desired concentration and V2 the desired volume. Culture flasks were returned to incubation conditions immediately after cell passaging. After cell passaging flasks were returned to incubation conditions.

2.2 Plasmid DNA

2.2.1 Plasmid origins

Plasmids stocks were obtained from UCL, Invivogen or Novogen, unless cloned in house. All plasmid DNA concentrations were analysed using a nanodrop spectrophotometer (Thermo Scientific, Loughborough, UK). DNA coding for the scFv SM3E was obtained from Dr Enrique Miranda Rota (UCL Cancer Institute, London, UK). Table 2.1 shows the derivation of the construct and related literature.

Table 2.1 MFE-23 development, descriptions and associated mutations.

Protein Name	Mutations	Reference
MFE-23	scFv generated via phage display library against CEA.	(Chester et al., 1994)
hMFE-23	Humanised MFE-23: 28 residues altered to produce humanised resurfaced form.	(Boehm et al., 2000; Graff et al., 2004)
shMFE-23	Stabilised humanised MFE-23; Mutations in V_L N20T, S31P, W47L, M78V.	(Graff et al., 2004)
SM3E	Affinity matured and stabilised shMFE-23; Mutations in V_L N20T, S31P, F36L, W47L, S50L, M78V.	(Graff et al., 2004)

2.2.2 Restriction enzyme digestion

Restriction enzyme digestion of plasmid DNA for analysis or cloning purposes was carried out using enzymes from New England Biolabs (NEB) (MA, USA). Restriction digests were carried out using digestion buffers and conditions as recommended in the

manufactures instructions (see table 2.1). Unless otherwise stated digestions were incubated for one hour at 37°C in total reaction volumes of 25 μL.

2.2.3 Agarose gel electrophoresis

Unless otherwise specified agarose gel electrophoresis was carried out using 50 mL of 1% agarose (Sigma Aldrich) in 1x TAE (see appendices Buffers and Reagents). The solution was heated in a microwave until fully dissolved before addition of 3 μL of ethidium bromide and pouring into a Bio-Rad gel system and allowing to set. Gels were covered in TAE buffer before DNA loading buffer at 10x concentrate was added to DNA to make a 1x solution prior to gel loading. The running conditions were 10V/cm for 1 h and a molecular weight marker was included dependent on expected band size.

2.3 Plasmid amplification and purification

2.3.1 Plasmid amplification

DNA was transformed via electroporation using a Bio-Rad Micro Pulser electroporater. Top10 electrocompetent *Escherichia coli* stocks were thawed on ice for 10 min before 1 μL of pDNA was added and transferred to an electroporation cuvette, also on ice. Electroporation was carried out using on the EC2 setting. Room temperature 2TY media (See appendices Buffers and Reagents) with 1% glucose was added (1mL) immediately after electroporation and the cuvette transferred to incubation conditions at 37°c for 1 hr. Transformed Top10 *E. coli* cultures were streaked onto 2TY agar plates supplemented with 100 μg/mL ampicillin before a 24hr incubation at 37°C. Electroporation of TG1 electrocompetent *E. coli* was carried out as above.

2.3.2 Selection, amplification and purification of plasmid DNA

A single bacterial colony was selected from an inoculated 2TY agar plate supplemented with 100 μg/mL ampicillin using a sterile inoculation loop and used to inoculate 5 mL of 2TY media supplemented with 1% (v/v) glucose and 100μg/mL ampicillin in a 15ml falcon tube and incubated at 37°C for 24hrs with 225rpm

agitation in an Innova 4230 incubator (New Brunswick, Arlington, UK). A 100 μL/L inoculation was then added to an appropriate amount of ampicillin 2TY media and incubated at 37°C, 225rpm for 24 hrs. Plasmid DNA was purified using endotoxin-free gigaprep, midiprep or mini-prep kits from Qiagen (Manchester, UK).

2.3.3 Polymerase chain reaction (PCR)

PCR amplification of template DNA was carried out using Phusion Polymerase (NEB, Hertfordshire, UK) unless otherwise stated. Reaction mixture contained 4 μL of Phusion HF (5x) buffer, 0.4 μL of 10mM dNTPs 1 μL of both forward and reverse primers at 10μM, <1μg of template DNA, 0.2 μL of Phusion Polymerase and nuclease free ddH2O to a final volume of 20 μL. Reaction conditions were 98°C for 30 s, 35 cycles of; 68-72°C for 20-30 s (dependent on primers) followed by 30 s per kb of DNA extension with a final extension of 5 min at 72°C and an infinite hold at 4°C.

2.3.4 Preparation of bacterial glycerol stocks

A 0.5mL aliquot of overnight culture of transformed bacterial was added to 0.5mL of autoclaved 50% glycerol before storage immediately at -80°C. Glycerol stocks were thawed on ice and streaked on selection plates for overnight incubation for further use.

2.4 Transient transfection of suspension adapted HEK293F and CHO cells

2.4.1 Preparation of 25 kDa linear polyethylenimine

Polyethylenimine (PEI) (25 kDa, linear form) was obtained from Poysciences (Eppelheim, Germany). 100mg was added to 80 mL autoclaved ddH20. A 4M solution of HCL was added to reduce the pH to <3 and the solution allowed to stir using a magnetic stirrer for 1 h at room temperature. Once the solution was colourless a 4M solution of NaOH was added to return the solution to pH 7.2 and ddH20 added to make a final volume of 100 mL at 1 mg/mL of PEI. The solution was filter sterilised using a 0.22 μm syringe filter (Millipore, Darmstadt, Germany) in a sterile tissue culture hood and aliquoted into 1 mL Eppendorf tubes for storage at -80 °C.

After thawing, PEI solution was either used immediately or kept at 4°C for up to 1 week before disposal.

2.4.2 Preparation of branched 25 kDa polyethylenimine

Branched form 25 kDa polyethylenimine was obtained from Sigma Aldrich in viscous liquid form. Branched form PEI was dissolved in an appropriate amount of ddH20 to obtain a 1mg/mL solution (1 mL/L). The solution was heated to 60°C for 1 h with magnetic stirring, allowed to cool to room temperature and filter sterilised using a 0.22 μm filter (Millipore) before storage at 4°C.

2.4.3 Cell culture prior to transfection

HEK293F and CHO cells were seeded 24hrs prior to transfections in pre-warmed Freestyle serum-free media at a density of $0.5x10^6$ cells/mL. Transfections were carried out in either 30 mL media in 125 mL vented conical flasks and for large-scale transfections cells were seeded in 300 mL media in 1L vented conical flasks. Once cell densities reached $1x10^6$ cells/mL with over 90% viability and good single cell dispersion as determined via the Trypan Blue exclusion assay transfection was carried out.

2.4.4 Transient transfection

293Fectin-mediated transfection

In a 2 mL eppendorf 60 μL of 293fectin was added to 940 μL of Optimem and mixed by inversion. In a second eppendorf 30 μg of plasmid DNA (minimum DNA concentration 100 ng/μL) was added to Optimem to a final volume of 1 mL, mixed once by inversion and incubated at room temperature for 5 minutes.

After incubation the pDNA–Freestyle media solution was added drop wise to the 293Fectin solution, mixed once by inversion and incubated at room temperature for 20 minutes before being added drop wise directly to the cell culture whilst gently swirling the flask. The flask was returned to incubation conditions as described above.

25 kDa linear Polyethylenimine –mediated transfection

Freestyle media (to final volume 2 mL) was added to a 1.5 mL eppendorf followed by 30 μg of plasmid DNA via pipette. The solution was mixed twice by inversion and 60 μL of a 1mg/mL linear 25 kDa PEI solution was added drop wise via pipette. The transfection solution was vortexed briefly and incubated at room temperature for 10 minutes. The transfection solution was then added drop wise directly to the cell culture whilst swirling the flask gently. The culture was then returned to incubation conditions.

2.4.5 Transient transfection of HEK293F cells in PBS 3L reactor

A PBS3L SUT reactor vessel containing 2.4L of Freestyle media was allowed to equilibrate to 37°C before being seeded with from HEK293F cells at a density of $3x10^6$ cells/mL cultured in shake flasks as described in section 2.1.5.2. Initial seeding density was $0.3x10^6$ cells/mL with a typical four-day expansion time before transfection at $1x10^6$ cells/mL. Culture samples were removed using the PBS Biotech sample manifold. Reactor conditions were set at 37°C with 25RPM agitation, pH7.2 and >30% DO_2 maintained via PID control (Kim et al., 2013).

2.5 Hela cell transfection

2.5.1 Transfection for random integration into Hela cells

Hela cell culture ($1x10^6$ cells/mL) was seeded in 5 mL of supplemented DMEM at a density of 1x105 cells/mL in T25 Tissue culture flasks 24 hours prior to transfection to attain 70% confluency on the day of transfection. When cells reached 70% confluency they were transfected.

Plasmid DNA (pFUSE-SM3E-Fc) (5 μg) was added to 5 mL of DMEM supplemented with 2mM L-glutamine only in a 15 mL Falcon tube. The solution was vortexed briefly and incubated at room temperature for 5 minutes. 20 μL of a 1 mg/mL branched 25 kDa polyethylenimine was added to the solution before vortexing briefly and a further 5-minute incubation at room temperature.

The media in the cell culture flasks was aspirated and replaced with the transfection solution and cells were returned to incubation conditions. Control flasks were treated with 5mL DMEM supplemented with 2mM L-glutamine and 20μl of branched PEI as above. After 24 hours the media was aspirated and replaced with DMEM supplemented with 10% FCS, 1% penicillin and streptomycin (PenStrep) antibiotic, 2mM L-Glutamine and 150 μg/mL zeocin.

2.6 Stable transgene integration in CHO-F cells

Stable cell selection was carried out after transfection with pUCOE-SM3E-Fc using FreestyleMAX (Life Technologies, Paisley, UK) transfection reagent as per the manufacturer's instructions. Antibiotic selection was carried out using addition of 12.5 μg/mL puromycin (Invivogen, Toulouse, France) to media 48 hrs after transfection unless otherwise stated. Cells were maintained under selection conditions until untransfected control cells reached <10% viability and transfected cells recovered to >90% viability as determined by daily Trypan blue staining and counting. After selection, stable cells were stored in liquid nitrogen at a density of $1x10^7$ cells/mL.

2.7 Fed-batch fermentation of CHO cells

Fed-Batch culture of CHO cells was performed using either 1L shake flask culture flasks (Corning) or single-use pneumatic bioreactor from PBS Biotech Inc (CA, USA) with a working volume of 3L was used for scaled up cell culture.

2.7.1 PBS 3L Bioreactor fed-batch fermentation of CHO cells

Sterile polycarbonate gamma-irradiated SUT 3L units (PBS Biotech Inc, CA, USA) were seeded with CHO cells at a density of $0.3x10^6$cells/mL. Reactor conditions were set at 37°C with 25RPM pneumatic vertical wheel agitation, pH7.2 and >30% DO_2 maintained via PID control (Kim et al., 2013). Cells were maintained in a fed-batch mode of operation with 3% (v/v) EfficientFeed B (Life Technologies, Paisley, UK) daily from Day 4. Once cells reached the end of exponential growth phase temperature and agitation were reduced to 35°C and 20RPM respectively (Nam et al., 2008). Cells were harvested when viability dropped below 80% or after day 14.

2.7.2 Fed-batch shake flask culture of CHO cells

Shake flasks cultures (300mL) in 1L flasks with 0.22μM vented caps (Corning) were seeded at a density of 0.3-0.5x10^6 cells/mL in 50% CD-CHO/ 50% Excel CHO. Feeding was carried out from day 4 as above (1.7.2).

2.8 Media Bioprofiling

Media composition and cell culture metabolites were analysed by ion selective electrode potentiometry, amperometry, and enzymatic reaction-dependent biosensors in 1mL aliquots immediately after sample removal (BioProfile 400, Nova Biomedical, Inc., Waltham, MA).

2.9 Cell analysis and imaging

2.9.1 Automated cell counting

Where automatic cell counting was used a Vi-CELL XR automated cell counter (Beckman Coulter, High Wycombe, UK) was used to determine cell density and viability via the Trypan blue exclusion method. Vi-CELL parameters were min/max size 6-50μm and 50 repeated counts. Cell cultures were diluted in phosphate buffered saline (PBS) were necessary to ensure counting accuracy.

2.9.2 Particle size distribution

Particle size distribution was analyzed using the CASY Cell Counter Analyzer System (Roche, Basal, Sweden) with cell analyses parameters set to min/max 3-40μm, particles greater than 40μm where deemed to be cell and protein aggregates. Cell culture was diluted in phosphate buffered saline where appropriate to ensure accuracy in cell counting and particle distribution analyses.

2.9.3 Cell and fluorophore imaging

Green fluorescent protein (GFP) and red fluorescent protein (DS-RFP) expression was analysed using fluorescent microscopy on an Aiovert100 microscope and Axiocam from Carl Ziess limited. Briefly, a 10 μL solution of 1x10^5cells/mL was

added to a microscope slide chamber. Cell and fluorescence imaging was carried out using the appropriate filters to excite fluorophores (RFP – 554nm and GFP- 498nm.

Antibody localisation staining was carried out using SM3EL-Fc (1μg/mL) added to either Capan-1 cells or A375 CEA negative cells cultured in 6cm^2 tissue culture plates. The plates were incubated at 4°C for 1 h to prevent internalisation. Secondary staining of the scFv-Fc was achieved using an anti-murine IgG conjugated Alexaflour 488 (green) whilst the nucleus is stained using DAPI (blue). Images were captured using a Leica TCS SP Confocal Microscope (Leica, Milton Keynes, UK) and image analysis performed using ImageJ (with help from Dr Enrique Miranda Rota).

2.9.4 Flow cytometry

The CyAn ADP Flow Cytometer (Beckman Coulter, High Wycombe, UK) was used to determine transfection efficiency of reagents using a red fluorescent protein. HEK293 or CHO cells at a viable cell density of 2x10^6 cells/mL were removed from culture, centrifuged at 350*g* and re-suspended in PBS twice before a third re-suspension in chilled 70% ethanol and incubation on ice for 30 min. After fixing cells were re-suspended in PBS prior to loading onto the flow cytometer. Un-transfected control cells were loaded to achieve gating of flow cytometry data before analysis of DS-RED fluorescence at 558nm (R1) and 583nm (R2) of transfected culture samples. Flow cytometry data was analysed using FlowJo analysis software.

2.9.5 MTS Tetrazolium Assay (MTT Assay)

CEA-expressing capan-1 cells and CEA-negative Hela cells were plated out in a 96-well tissue culture plate (10,000 cells/well). After 24 hrs 1μg of SM3EL-Fc was added to each well and the plate was incubated at 4°C for 1 hr. Rabbit complement (AbD Sertech) was added (11μL/well) and the plate incubated for a further 1 h at 37°C followed by MTS solution (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Cell Titre 96 Aqueous One Solution, Promega) assay substrate (12μL/well) and an incubation for 30min at 37°C. Analysis of developed colour was performed using a Varioskan Flash plate reader (Life Technologies, Paisley, UK) at 490nm.

2.9.6 Quantification of protease activity in supernatants

Protease activity in cell culture supernatant and flow-through from column-based chromatography was determined using the fluorescence resonance energy transfer (FRET) protease assay (Life Technologies, Paisley, UK) according to the manufactures instructions. Briefly, 100µL of fluorescein labeled casein stock solution (10mg/mL) or assay buffer as blank was added to each well of a black Nunc 96-well plate (Thermo Scientific). Supernatant (50µL) was added to wells in triplicate and incubated for 20-30min at room temperature before addition of 50µL 5% (v/v) trinitrobenzene sulfonic acid (TNBSA) solution in methanol and a further incubation of 20min at room temperature to allow color to develop. The assay plate was read in a Varioskan Flash plate reader in fluorescence mode at 485/538nm. Activity was quantified against a standard curve of trypsin with known proteolytic activity, 15525 Unit/mg (1 Unit defined as a change in absorbance at 253 nm of 0.001 per minute at 25 °C, pH 7.6 in a reaction volume of 3.2 mL of Na-Benzoyl-L-Arginine Ethyl Ester Solution (BAEE) (Papaioannou and Liener, 1968).

2.9.7 β-Galactosidase activity

β-Galactosidase activity was analysed using the β-Gal Assay Kit from Invitrogen (K1455-01 Version F) according to the manufactures instructions. In brief, the media was aspirated from T25 flasks of transfected cells followed by washing with 1xPBS and trypsinising into PBS solution. The cells were counted and normalised to $1x10^5$ cells/mL in 1 mL. The solution was centrifuged at 250*g* for 5 min, the supernatant aspirated and the cells re-suspended in 100µL lysis buffer. Cells were lysed by freeze-thawing on dry ice and heating to 37°C twice and the cell debris pelleted at 4122*g* for 5 min. 20µl of the soluble lysed cell material was added to a fresh tube along with 70µL of ONPG substrate and 200µL of cleavage buffer before incubating at 37°C. Once colour had developed (20-25min), 500µL of stop solution was added and 200µL of each sample added to each well of a 96-well plate for absorbance analysis at 420nm in a Varioskan Flash plate reader (Life Technologies) and compared to control conditions.

2.9.8 Quantitative qPCR for detection of CHO cell genomic DNA in supernatant.

2.9.8.1 Genomic DNA isolation

CHO cell culture (2 mL) at a cell density of ≥5x10^6 /mL were pelleted by centrifugation at 200*g* for 5 min and the supernatant aspirated. The cell pellet was re-suspended in 1mL TRIzol reagent (Qiagen) /5x10^6 cells and lysed by aggressive pipetting. After homogenization the samples were incubated for 5 minutes at RT before 200 μL of chloroform was added per 1 mL of TRIzol reagent. The tubes were shaken vigorously for 15 seconds and incubated at 30°C for 2 minutes.

The samples were separated into aqueous and organic phases by centrifugation at 12,000*g* for 15 minutes at 4°C. The aqueous phase was removed by pipette and discarded. Absolute ethanol (300 μL/ 1 mL TRIzol) was added and incubated for 2 min at room temperature before centrifugation at 2000*g* for 5 min to pellet DNA. The supernatant was decanted and the pellet washed twice using 100 mM sodium citrate in 10% ethanol (2 mL) for 30 min at room temperature and subsequently pelleting by centrifugation before a final wash in 70% ethanol and re-suspension in TE buffer.

2.9.8.2 qPCR analysis of genomic DNA contaminant in supernatant

Contaminating DNA was quantified by real time PCR performed in a Realplex4 Mastercycler EPgradient S (Eppendorf, Stevenage, UK) using the following conditions: initial heat denaturation at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles each of 95°C for 15s and 60°C for 1 min. Sample preparation required recovery of cell culture supernatant via centrifugation at <500*g,* followed by removal of the supernatant to a fresh tube and centrifugation at13000*g* for a further 5 min and 0.22μm filtration. The supernatant was analyzed in 3μL samples using previously isolated and quantified genomic DNA. Unless otherwise stated the reaction mix contained 3μL of supernatant sample in a 25μL reaction mixture of ITAQ SYBR with a final concentration of 5 nM of each primer. Primers directed against genomic DNA isolated from *Critcetulus griseus* (CHO) were synthesized by IDT (Integrated DNA Technologies, Glasgow, UK). Primer sequences were: Sense 5'ACAGGTTTCTGCTTCTGGCT and Anti-sense 5'CATCAGCTGACTGGTTCACA. The reaction mix contained 3μL of sample in a

25μL reaction mixture of ITAQ SYBR with a final concentration of 5 nM of each primer.

2.10 Chromatography

2.10.1 Hightrap protein A chromatography

Proteins with Fc fragments were captured from mammalian suspension cultures after solid liquid separation. Cultures of ≤300mL were centrifuged at 400x*g* for 5 min to remove cells/debris and the supernatant was removed and filtered via 0.22μm vacuum filtration. The filtrate was concentrated where appropriate and dialysed into 20mM sodium phosphate using tangential flow filtration (Labscale TFF, Millipore) with Pellicon filtration cassettes (Millipore) of an appropriate pore size. Two volumes of buffer exchange were used per total supernatant volume to gain full dialysis.

Proteins were captured using a HiTrap rProtein A ff 1mL column from GE Healthcare (Cat. Num. 17-0402-01) and a Bio-Rad Econopump chromatography pump. Unless otherwise specified flow rates were set at 1 column volume (CV)/min. The column was washed using 10 column volumes of 20mM sodium phosphate and the dialysed supernatant was applied to the column. A 5 CV wash using binding buffer preceded elution in 0.1M sodium citrate (pH3) using 60μL/mL of Tris-HCL buffer (pH 8.4) to neutralise protein solutions. Proteins were quantified by absorbance at 280nm using a nanodrop spectrophotometer.

2.11 Radial flow chromatography operation

A scale down RFC column with bed volume of 5 mL and height of 6cm was used with either 40μm or 200μm pore size frits (Proxcys, Nieuw-Amsterdam, Netherlands) (Fig 1.A). The column was packed with iminodiacetic acid (IDA) Chelating Cellthru^{TM} BigBead agarose resin (Sterogene, Carlsbad, CA) as per the manufacturer's instructions. Column conditioning was performed to remove air bubbles with 6x column volumes (CV) PBS (pH 7.2). The resin was charged with 5CV 100mM nickel sulphate and equilibrated with 0.5M NaCl/ 0.5x PBS/ 10mM imidazole. An operational flow rate of one CV/minute was used for column loading, achieved using a peristaltic pump (New MBR, Zurich, Switzerland). Whilst frit pore

sizes of both 200μm and 40μm were studied, the 200μm pore frit was used routinely. Non-specifically bound proteins were removed via wash buffer (40mM imidazole/ 0.5X PBS/ 0.5M NaCl) before elution with 200mM imidazole/0.5X PBS/ 0.5M NaCl. The column was stripped and regenerated after each use using 50mM NA-EDTA and stored in 20% (v/v) ethanol.

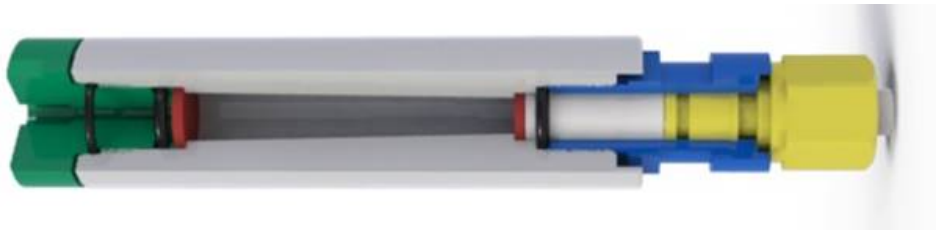


Figure 2.1 Cross section of microRFC 5mL radial flow column (image courtesy of Proxcys). The microRFC column has a bed height of 6cm and volume of 5mL.

2.12 Column-reactor integration and harvest

In-line integration of RFC was achieved via welding the column tubing directly to the reactor harvest line using Masterflex C-FLEX tubing (Cole-Palmer, London, UK) with a sterile tube welder (GE Healthcare, Buckinghamshire, UK). Valve and pump operation was used to control flow from the reactor (Valves 1 and 3 open), followed by wash, elution and regeneration buffers (Valves 2 and either 3 or 4 open) (Fig 1.B). Cells were buffered to IMAC binding conditions by addition of 4x concentrated IMAC buffer to the reactor prior to passage directly through the column in 3x1L batches. The column was eluted and regenerated between batches as described above.

2.13 Model His-tagged protein recovery

A His-tagged scFv (SM3E) (Graff et al., 2004) was expressed and purified from *Pichia pastoris* as previously described (Tolner et al., 2006) and used as the test protein for recovery from cell culture media. Ultraviolet light absorbance and conductivity were analyzed by linking the RFC to an AKTA Prime Plus (GE Healthcare, Buckinghamshire, UK). Eluted fractions were resolved by 10% (v/v) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), stained for 1 h with Coomassie Brilliant Blue (Sigma Aldrich, St. Louis, MO) and destained

until clear using a mixture of 10% (v/v) glacial acetic acid, 30% (v/v) methanol and 60% (v/v) double distilled water (ddH_2O).

2.13.1 Fast Protein Liquid Chromatography (FPLC) separation of proteins.

FPLC separation of recombinant proteins was carried out using a Superdex200 75mL column and an AKTA Prime Plus FPLC machine from Amersham Biosciences (Buckinghamshire, UK). Protein samples at a minimum concentration of 100μg/mL were loaded on to the column in maximum 2mL quantities using the manual loop injection mode. Running conditions were 1 mL/min and 1.5MPa pressure limit, optical density was measured at 280nm for protein detection and fraction collection was set at 1mL.

2.14 Protein storage

After elution from chromatographic capture proteins were dialysed into PBS overnight, aliquoted into appropriate volumes and stored at -80°C unless other wise specified. Protein dialysis was carried out using Slide-A-Lyzer dialysis cassettes (ThermoFisher) with pore size restricted to between 10X lower than the size of the target protein IE NMWC of 10000 for a protein of 100 kDa. Proteins were dialysed in 5L of PBS solution overnight with magnetic stirring at 4°C.

2.15 Biophysical analysis of recombinant proteins

2.15.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were cast in a Bio-Rad Handcast dock using the reagents specified in Table 2.2. Protein solutions were diluted to 0.25 mg/mL with PBS where necessary prior loading. A 4X concentrated reducing buffer was added to samples prepared for SDS-PAGE under reducing conditions. Samples where heated in a PCR machine (Biometra Personal, Göttingen, Germany) for 5 min at 100°C. Reduced protein solutions were added to the gel and the electrophoresis carried out at 150V for 1 h. SDS-PAGE gels were washed with ddH2O and stained for 1 h with Coomassie Brilliant Blue (Sigma Aldrich, St. Louis, MO) with gentle agitation on a rocking platform. Coomassie stained gels were destained until clear using a mixture of 10%

(v/v) glacial acetic acid, 30% (v/v) methanol and 60% (v/v) double distilled water (ddH_2O).

Table 2.2 SDS-PAGE gel solutions

Reagent	4% Stacking Gel	10% Resolving Gel
ddH_20	3.6 mL	3.2 mL
30% Acrylamide (Protogel)	0.67 mL	2.67 mL
1.5M Tris-HCL pH 8.8	na	2 mL
1.0M Tris-HCL pH6.8	0.625 mL	na
10% SDS	50 μL	80 μL
10% Ammonium Persulphate	50 μL	80 μL
TEMED	5 μL	8 μL

2.15.2 Western Blotting

Purified proteins were diluted to 1μg/mL prior to being resolved by SDS-PAGE as above and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) using 110V for 1 h. Positive control proteins included His-tagged CEA (R&D, Abingdon, UK). Membranes were blocked with 5% (w/v) skimmed milk powder in 0.1% (v/v) PBS and probed with murine anti-His tag (Qiagen, Limburg, Netherlands) or an anti-CEA antibody (Pedley et al., 1987). Bound antibodies were detected with HRP-linked secondary anti-mouse-HRP conjugated antibody (R&D, Abingdon, UK) and developed using Luminata Classico Western HRP substrate (Millipore, Darmstadt, Germany).

2.15.3 Differential scanning fluorimetry

DSF was used to monitor IgG and scFv-Fc unfolding during exposure to a temperature gradient. A 96-white well PCR plate was used during DSF experiments (Bio-Rad Laboratories, Hercules, CA), with each well containing 22.5 µL of IgG or scFv-Fc sample in PBS and 2.5 µL of 10x SYPRO Orange solution (Life technologies) that had been diluted from the purchased 5000x stock in water. A Realplex4 Mastercycler EPgradient S RT-PCR machine (Eppendorf, Stevenage, UK)) was used to record fluorescence changes during DSF measurement in FRET scanning mode. Samples were incubated at 15 °C for 4 min before exposure to a temperature gradient of 20 to 95 °C in 0.5 °C increments, with an equilibration time of 30 s at each temperature followed by a fluorescence read. Protein unfolding was reported as the midpoint transition temperature at which hydrophobic regions become exposed (Tm). Mean Tm values were determined using first-order derivative curves of triplicate experiments with reference to a blank buffer as background.

2.15.4 Enzyme Linked Immunosorbant Assays

ELISA based quantification of CEA was carried out using a double antibody sandwich ELISA method. A 96-well plate (Life Technologies) was coated with 10 µg/mL polyclonal rabbit anti-CEA (UCL Cancer Institute, London). After washing with PBS twice, wells were blocked with 200 µL of 5% (w/v) skimmed milk in PBS. Plates were washed as before and samples were added along with a serial dilution of His-tagged CEA (R&D, Abingdon, UK) to form a standard curve. After washing, 1 µg/mL murine anti-CEA was added for 1 h and detected using anti-mouse-horseradish peroxidase (HRP) conjugate (R&D, Abingdon, UK) diluted 1:4000 in PBS, using 0.5 mg/mL o-phenylenediamine dihydrochloride (Sigma–Aldrich) in phosphocitrate buffer for development. Intensity was analysed by absorbance at 490 nm in a Varioskan Flash platereader (Life Technologies). All incubations were carried out at room temperature.

ScFv-Fc concentrations and activity were analysed by either double antibody sandwich (DAS) or plate trapped antigen (PTA) ELSIA formats. In both cases purified and quantified recombinant protein was used as a standard curve.

2.15.4.1 DAS-ELISA format

- A 96-plate was coated with 100 μL/well of a 1/4000 dilution in PBS of goat anti-human IgG-Fc (Jackson, 109-005-098) for 1 h. The assay plate was flicked off and blocked for 1 h as above. Samples were added (100 μL/well) and incubated at room temperature for 1 h before washing as above. A secondary anti-mouse-HRP conjugate as described above was used to report fluorescence quantification using a 1/4000 dilution with incubation for 1 h at room temperature followed by development using OPD and absorbance measurement as above.

2.15.4.2 PTA-ELISA format

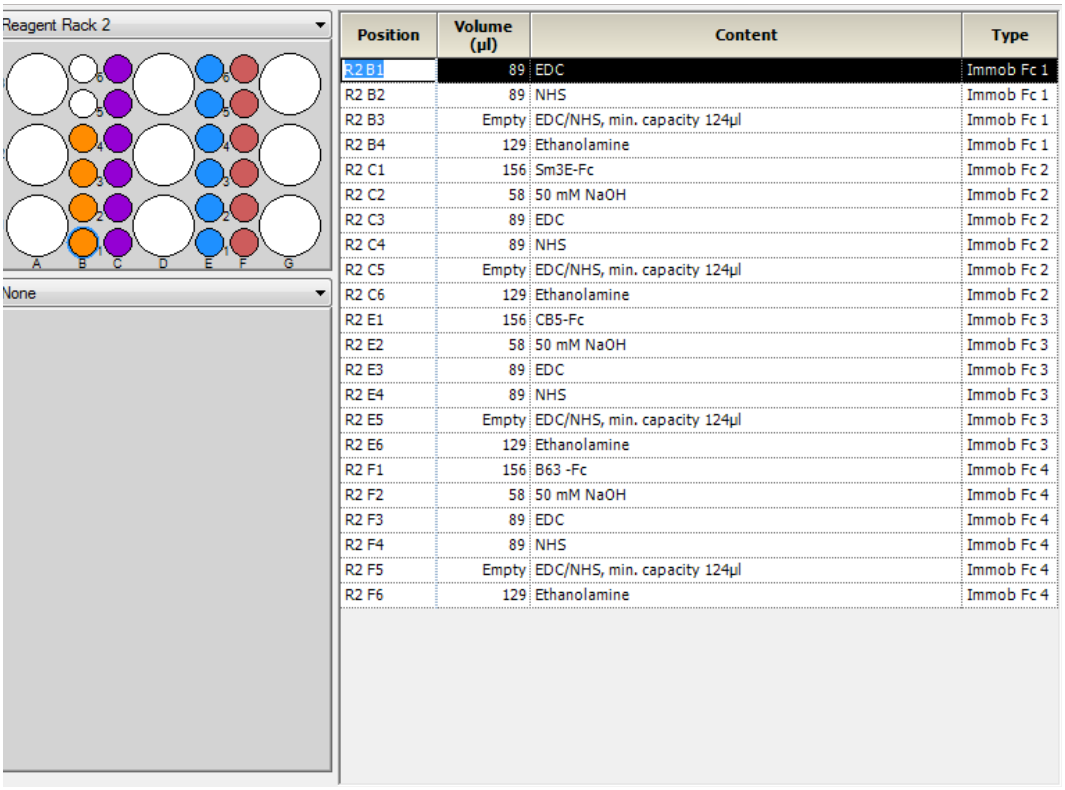
- A 96-well plate was coated overnight at 4°C with a 1 μg/mL dilution of recombinant NA1 (Produced in house accoding to Sainz-Pastor et al., 2006; Tolner et al., 2013) in PBS. The plate was blocked as above and the assay samples added from a titration plate and incubated for 1 h at room temperature. The assay was detected and reported using the anti mouse Fc-HRP conjugate as described above.

2.16 Surface Plasmon Resonance

Surface plasmon resonance (SPR) spectroscopy was performed using a T200 enhanced Biacore (GE Health Care Life Sciences, Amersham, UK). All solutions used were 0.22 μm filtered prior to use, however not degassed. Analysis of affinity and binding kinetics was carried out via immobilisation of the antibody as ligand where possible using CM5 chip (carboxymethylated dextran covalently attached to a gold surface) and 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)/ N-Hydroxysuccinimide (NHS) based covalent amine coupling chemistry according to the manufactures instructions.

In brief, the CM5 chip surface was activated using EDC/NHS prior to immobilisation of proteins prepared in solutions of 1-10 μM on single flow cells leaving flow cell one blank as reference. The ligand was past over the flow cell at a rate of 5-10 μL/min before 1M Ethanolamine hydrochloride-NaOH (pH 8.5) was used to deactivate excess

binding groups. See plate layout according to BIAcore CM5 immobilisation wizard (Figure 2.2 Biacore CM5 Chip immobilisation plate set up according to template protocol.



Position	Volume (µl)	Content	Type
R2 B1	89	EDC	Immob Fc 1
R2 B2	89	NHS	Immob Fc 1
R2 B3	Empty	EDC/NHS, min. capacity 124µl	Immob Fc 1
R2 B4	129	Ethanolamine	Immob Fc 1
R2 C1	156	Sm3E-Fc	Immob Fc 2
R2 C2	58	50 mM NaOH	Immob Fc 2
R2 C3	89	EDC	Immob Fc 2
R2 C4	89	NHS	Immob Fc 2
R2 C5	Empty	EDC/NHS, min. capacity 124µl	Immob Fc 2
R2 C6	129	Ethanolamine	Immob Fc 2
R2 E1	156	CB5-Fc	Immob Fc 3
R2 E2	58	50 mM NaOH	Immob Fc 3
R2 E3	89	EDC	Immob Fc 3
R2 E4	89	NHS	Immob Fc 3
R2 E5	Empty	EDC/NHS, min. capacity 124µl	Immob Fc 3
R2 E6	129	Ethanolamine	Immob Fc 3
R2 F1	156	B63 -Fc	Immob Fc 4
R2 F2	58	50 mM NaOH	Immob Fc 4
R2 F3	89	EDC	Immob Fc 4
R2 F4	89	NHS	Immob Fc 4
R2 F5	Empty	EDC/NHS, min. capacity 124µl	Immob Fc 4
R2 F6	129	Ethanolamine	Immob Fc 4

Figure 2.2 Biacore CM5 Chip immobilisation plate set up according to template protocol.

Kinetic affinity analysis was carried out using starting sample concentrations of 200-400nM with ½ dilutions in 1HBS-EP+ (10mM HEPES, 150mM NaCl, 0.05% v/v Surfactant P20, pH 7.4 10mM HEPES, 150mM NaCl, 0.05% v/v Surfactant P20, pH 7.4) to allow for multi-cycle kinetics. Flow rate was 30µL/min with association and disassociation times of 300s. The chip surface was regenerated using 10mM Glycine-HCL, pH2.5. Affinity analysis was carried out using the BIAevaluate software with global model fitting via Boltzmann fitting.

2.17 Design of Experiments

A central composite design (CCD) was used for optimization of buffer conditions for His-tagged CEA Ni-IMAC binding. Three variables were investigated with parameters based on standard IMAC binding buffers; (A) pH, (B) NaCl concentration

(mM) and (C) imidazole concentration (mM). Clarified supernatant (100mL) containing His-tagged CEA was adjusted to the appropriate buffer composition and randomized using the design matrix prior to capture using a 5mL HisTrap column (GE Healthcare, Buckinghamshire, UK). CEA yield was assessed via ELISA and process interactions were analyzed using Design-Expert 8 (Stat-Ease, Inc, MN. USA).

Table 2.3 General reagents and buffers

Buffer/reagent name	
SDS-PAGE running buffer	250mM Tris Base, 1.92M Glycine, 1% SDS solution, pH 8.3
Western blot transfer buffer	250mM Tris Base, 1.92M Glycine, 1% SDS solution, pH 8.3
Protein A elution buffer	100mM Citric acid, pH 2.7
Protein A binding buffer	20mM Sodium Phosphate, pH 7.2
Protein A wash buffer	20mM Sodium Phosphate, pH 7.2
Protein A Neutralisation buffer	1M Tris-HCL, pH 9.0
IMAC binding buffer	0.5M NaCl/ 0.5x PBS/ 10mM imidazole.
IMAC	100mM Nickel Sulphate
IMAC elution buffer	200mM imidazole/0.5X PBS/ 0.5M NaCl
IMAC wash buffer	0.5M NaCl/ 0.5x PBS/ 40mM imidazole
ELISA development buffer	0.1M sodium citrate, 40mg/100mL

	Ortho-phenylenediamine
HBS-EP+	0.1 M HEPES, 1.5 M NaCl and 0.5% v/v Surfactant P20 pH 7.4
PBS-Tween	1x PBS, 0.05% (v/v) Tween
Phosphate buffered saline (PBS)	NaCl 137mM, KCL 2.7 mM, Na2HPO4 10 mM, KH2PO4 1.8 mM
Reducing buffer	2.5mL MilliQ, 2.5mL 1MTris-HCL (pH6.8), 4.0mL Glycerol, 0.8g SDS, 0.8mL β-Mercapto-ethanol, 0.4mg Bromophenol Blue.

Chapter 3 Development of a rapid and cost effective recombinant protein expression system in mammalian cells using transient lab scale production

3.1 Introduction

Industrial production of complex transgenic glycoproteins such as therapeutic antibodies or Fc conjugates currently takes place in stably transfected mammalian cells. This ensures the correct structure and high expression of the protein required for therapy, as described in section 1.6.1. However, the process of stable clone selection is laborious and time consuming (Backliwal et al., 2008c) compared to transient gene expression (TGE) which can provide significant advantages when rapid production is required (Wurm, 2004).

TGE allows recombinant protein to be obtained a matter of days after transfection, rather than weeks as in the case of stable gene expression. Use of TGE early in protein development processes thereby provides a means for rapid protein expression with low cost and time constraints. Assessment of pre-clinical performance can then be achieved using small amounts of recombinant protein. Furthermore, TGE can be used to predict recombinant protein expression levels in stable cells (Bentley et al., 1998; Diepenbruck et al., 2012; Pybus et al., 2014b). TGE is therefore often used to produce the first quantities of novel proteins prior to committing to stable cell selection allowing more efficient product screening (Agrawal and Bal, 2012).

The choice of host system for expression of recombinant proteins is important to ensure correct protein function and productivity. Suspension adapted Chinese Hamster Ovary (CHO) cells are currently the industry standard for recombinant glycoprotein production due to regulatory familiarity and high yields. It would therefore be advantageous to use CHO cells as both preliminary and endpoint expression systems for a seamless developmental transition from the lab bench. However, the Human Embryonic Kidney (HEK293) cell line has also been adapted to serum-free suspension growth and is preferentially utilised for TGE due to the higher transfection efficiencies (Sun et al., 2008).

Both cell types can be transfected using a multitude of reagents and scales with varying limitations, associated cost and success. The scalable nature of TGE enables protein production at a range of volumes to meet requirements; however it is not yet clear what effects scale has on the subsequent cell growth, gene expression and protein stability.

The work in this chapter was designed to develop a system for rapid, scalable and cost effective production of recombinant proteins using a previously described scFv-Fc as a production model (Table 2.1). Unlike whole monoclonal antibodies, which have complications in terms of heavy chain to light chain ratios and the co-transfection using multiple vectors, scFv-Fc vectors are simple constructs that easily model the transfection behaviour of their larger counter parts. Comparison of transient gene expression in suspension adapted CHO and HEK293 cells and further optimisation and comparison of the performance of two transfection reagents was utilised to assess performance during scale up and yield of recombinant protein. Characterisation of the scFv-Fc was carried out to investigate the effect of scale on protein stability and to evaluate the model protein as a potential therapeutic candidate.

Aims

Develop a transient mammalian expression system for rapid, scalable and cost effective production of recombinant proteins for use in laboratory scale production and characterise resulting products

Objectives

- Construct a plasmid for scFv-Fc expression in mammalian cells
- Develop a system for transient expression of scFv-Fc comparing CHO-S and HEK293F cells
- Optimise transfection protocols for cost efficient protein expression
- Establish the effects of transfection scale-up on cell growth, productivity and protein stability
- Characterise the affinity and *in vitro* binding of scFv-Fc to the target antigen

3.2 Results

3.2.1 Construct a plasmid for scFv-Fc expression in mammalian cells

3.2.1.1 Design and cloning of SM3EL-Fc expression cassette

Recombinant DNA coding for the single chain variable fragment, SM3E (Table 2.1), was kindly provided by Dr. Enrique Miranda Rota. The scFv was fused to the Fc portion of a murine IgG2a antibody using a pFUSE-mIgG2A plasmid vector from Invitrogen (Toulouse, Fr), also containing the interleukin 2 signal sequence ensuring secretion of the protein. PCR amplification primers were designed to amplify the scFv fragment (757bp) and add the restriction sites 5' EcoRI and 3' BglII to enable simple complementary end ligation into the multiple cloning site of the vector.

Primer sequences;

Sense (Fwd): GCATACGAGCTGAATTCGATGGCTCAGGTGAAGCTGGAACA
Antisense (Rvs): CCTCTGGGAGATCTTTTGATCTCCAGCTTGGT

N.b. Underlined letters indicate insertion of restriction sites. Sense (Fwd) inserts EcoRI and Antisense (Rvs) inserts BglII sites.

The scFv fragment was amplified via PCR (Section 2.3.3) and double digested, along with the murine Fc expression vector, using BglII and EcoRI. The resulting fragments were resolved via agarose gel electrophoresis, excised and purified to obtain clean DNA. Ligation was successful using a 3:1 molar ratio (vector to insert) and the resulting plasmid DNA was transformed via electroporation into Top10 *E. coli* before incubation on 100μg/mL ampicillin-2TY selection plates overnight at 37°C. A total of 12 colonies were selected and grown in 5mL ampicillin-2TY media overnight prior to isolation of plasmid DNA. An analytical restriction digest of pDNA was performed using BglII and EcoRI with expected fragment sizes of 4189bp and 751bp, clone 6 produced DNA bands with the correct pattern (Figure 3.1**Error! Reference source not found.**). The scFv-Fc coding region of the plasmid was sequenced successfully and no mutations were incorporated during the cloning process.

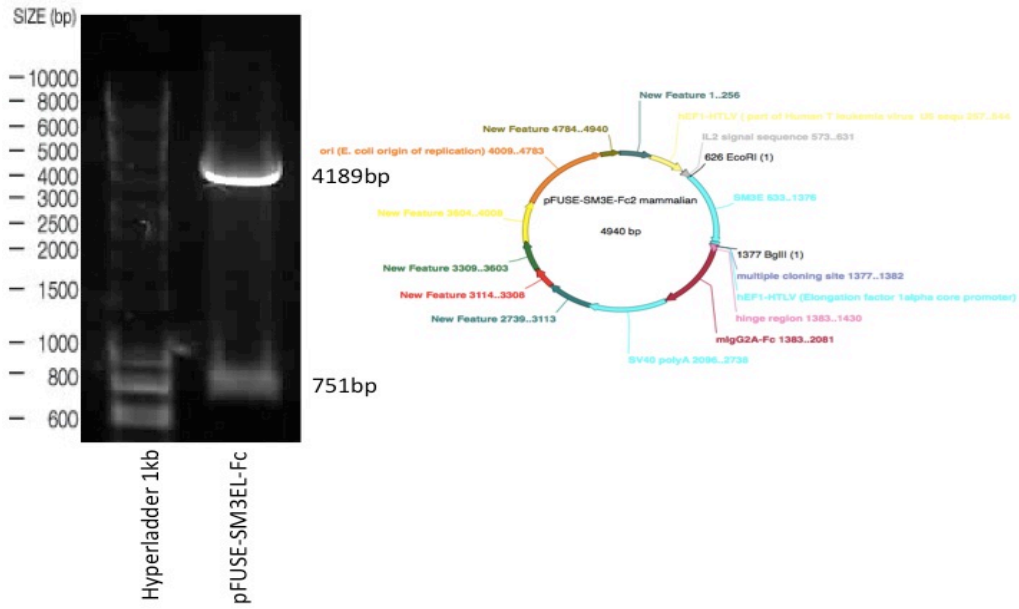


Figure 3.1 Restriction digest of pFUSE-SM3EL-Fc expression plasmid. DNA coding for an scFv was purified and ligated into an Fc fusion vector to produce an scFv-Fc conjugate. Successful ligation of the vector and insert was confirmed by analytical restriction digest and 1% agarose gel electrophoresis. A combination of EcoRI and BglII was used to cut the expression vector resulting in two bands at the expected sizes of 4189bp and 751bp. A 1Kb Hyperladder (Bioline) was included as a molecular weight marker.

The plasmid DNA was amplified via growth of transformed TOP10 *E. coli* in a volume dependent on the quantity of DNA required. For large-scale transfection purposes plasmid DNA was generated using an endotoxin-free Gigaprep kit from Qiagen using 2.5L bacterial cultures. After optimisation this yielded as much as 5mg of endotoxin free pDNA suitable for transfection at bioreactor scale.

3.2.1.2 Preliminary transient expression of SM3EL-Fc in HEK293F and CHO cells

HEK293F and CHO cells were transfected using polyethylenimine (PEI) in 30mL cultures that were maintained under incubation conditions for 7 days (Figure 3.2 A/B). Samples (0.5mL) were removed daily, centrifuged and the supernatant stored at -80°C. On day 7, cells were separated from the supernatant via centrifugation and filtration before a 1mL protein A mAbSelect chromatography column was used to capture scFv-Fc. The eluted protein was dialysed into PBS over night before being quantified by UV absorbance at 280nm using a nanodrop spectrophotometer as well

as separation and visualisation using 10% SDS-PAGE (Figure 3.2 D). Under reducing conditions the product resulted in a single band at the expected size (55 kDa) indicating that no product breakdown had occurred during expression and purification from either CHO or HEK293 cells. Secreted protein in the supernatants was quantified using a double antibody sandwich enzyme linked immunosorbant assay (DAS-ELISA) based on anti-kappa light chain and anti-murine Fc HRP conjugate antibodies. The ELISA was quantified against a standard curve generated with the purified scFv-Fc of known concentrations. Expression over the 7-day period reached a maximum of 8.65µg/mL in HEK293F cells and ~3.8ug/mL in CHO cells (Figure 3.2 C), with a total yield of 0.27mgs and 0.19mgs respectively after dialyses into PBS.

Preliminary expression of SM3EL-Fc confirmed that the plasmid construct successfully produced protein expression and that the product was quantifiable in supernatants by ELISA. Whilst expression of the protein was greater in HEK293F cells it remained low in both systems.

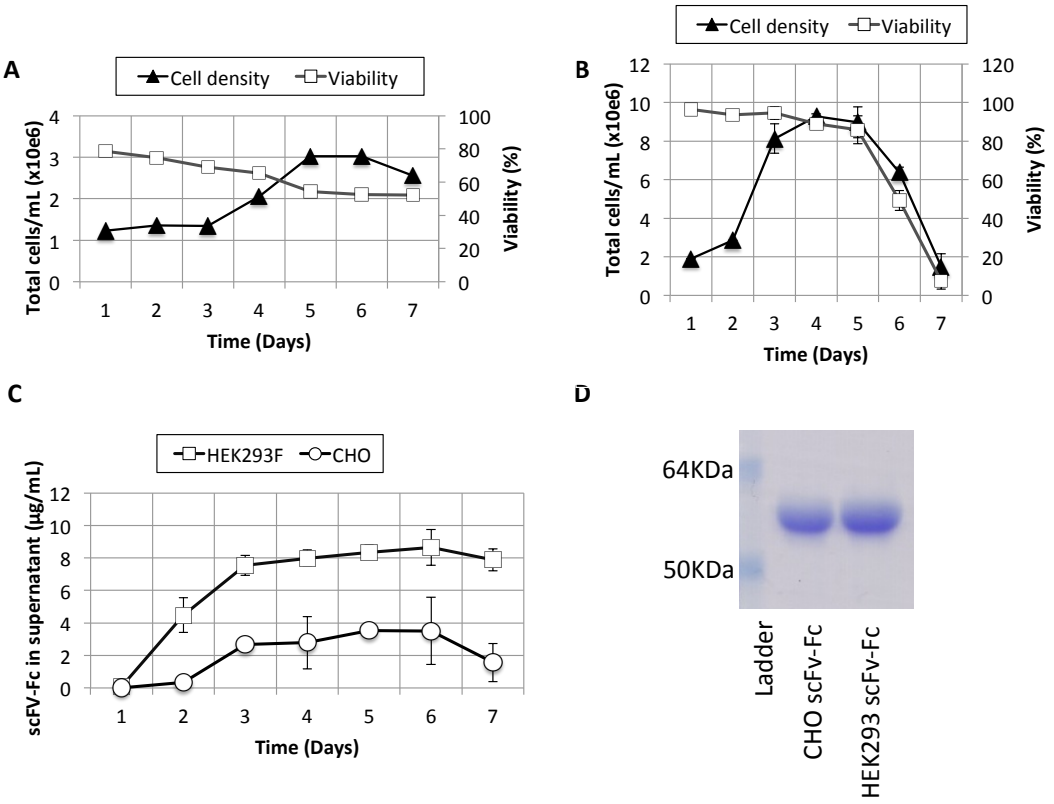


Figure 3.2 Preliminary expression of SM3EL-Fc in suspension HEK293F and CHO cells. 30mL cultures of HEK293F and CHO-S cells were transfected at a cell density of $1x10^6$ cells/mL using PEI. The cell growth and viability was assessed daily using the Trypan blue assay. Protein expression over 7 days was assayed using a

DAS-ELISA. Proteins from both flasks was resolved by 10% SDS-PAGE. A) HEK293F cell growth and viability B) CHO cell growth and viability C) Transient expression of SM3EL-Fc by HEK293F and CHO cells as determined by DAS-ELISA D) SDS-PAGE resolution of purified SM3EL-Fc showing CHO and HEK293 cell expressed SM3EL-Fc next to a PageRuler molecular weight marker. The molecular weight of the protein is 55 kDa. Error bars represent 1 standard deviation away from the mean (Biological and technical replicates = n3).

3.2.2 Develop and optimise a system for transient expression in either CHO or HEK293 cells

3.2.2.1 Polyethylenimine mediated transfection efficiency of HEK293F and CHO-S cells

Maximal yield of a protein is dependant on the percentage of cells successfully transfected, hence the transfection efficiency of CHO and HEK293F cell lines was determined via expression of a green fluorescent protein (GFP) reporter gene. Analysis of GFP expression by cells was achieved via fluorescence associated cell sorting (FACS) at 24 and 48 h post transfection with PEI to allow for a full cell cycle to take place. The HEK293F cell line showed a greater GFP positive population than CHO cells at both time points (at 24 h HEK293F 23.4% vs CHO 0.25% and at 48 h HEK293F 24% vs CHO 1.63%) (Figure 3.3). These results confirmed previous findings in the literature and determined the choice of expression system used for further transient expression of the scFv-Fc.

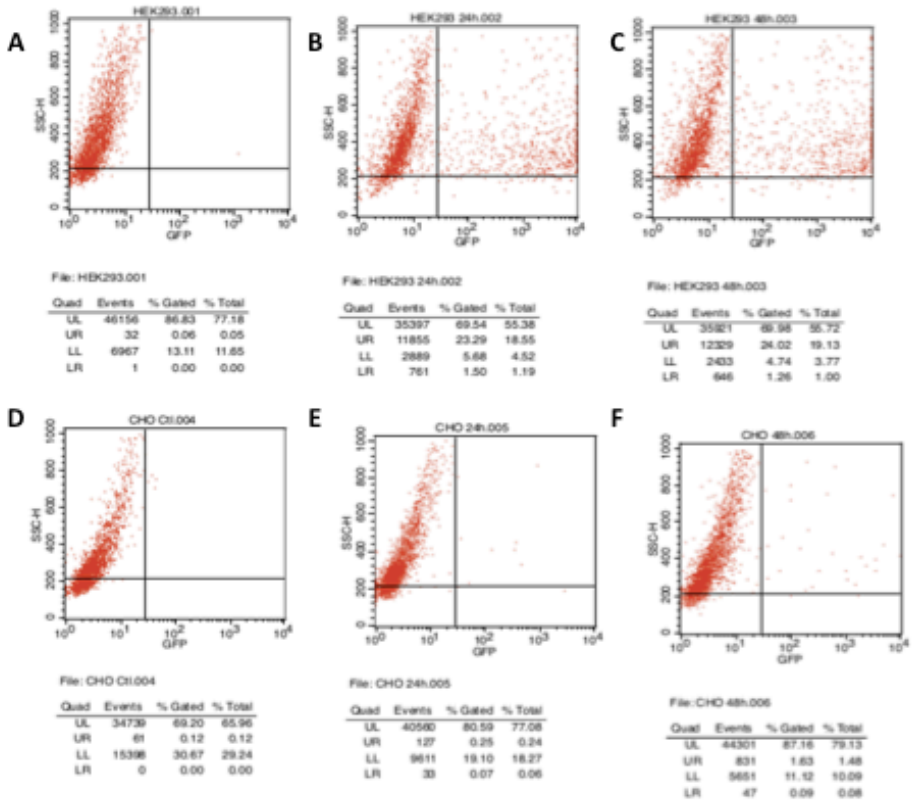


Figure 3.3 Polyethylenimine-mediated transfection efficiency of CHO and HEK293F cells expressing green fluorescent protein. CHO and HEK293F cells were transfected with pDNA coding for Green fluorescent protein (GFP) using PEI. GFP expression was assayed after both 24 and 48 h post transfection and compared to non-transfected control cells. A) HEK293 non-transfected control B) HEK293F 24 h post transfection C) HEK293F 48 h post transfection D) CHO non-transfected control E) CHO 24 h post transfection F) CHO 48 h post transfection.

3.2.2.2 Comparing efficiency of HEK293F transfection with polyethylenimine and 293Fectin

The cost and performance of transfection reagents can vary substantially. Here two reagents based on a liposomal transfection method were compared for transfection efficiency. Polyethylenimine is one of the least expensive reagents whilst 293Fectin is at the top of the price spectrum. HEK293F cells were transfected with a red fluorescent reporter protein (RFP) in shake flask cultures of 30mL according to the manufactures instructions (293Fectin) or previously published methods (PEI – Durocher et al., 2002). 293Fectin showed improved transfection efficiency compared to PEI mediated transfection after 24 h, 39.03% and 5.66% respectively. However there was also notable cytotoxicity and change in cell shape within hours of 293Fectin

addition compared to little change associated with PEI mediated transfection (Figure 3.5).

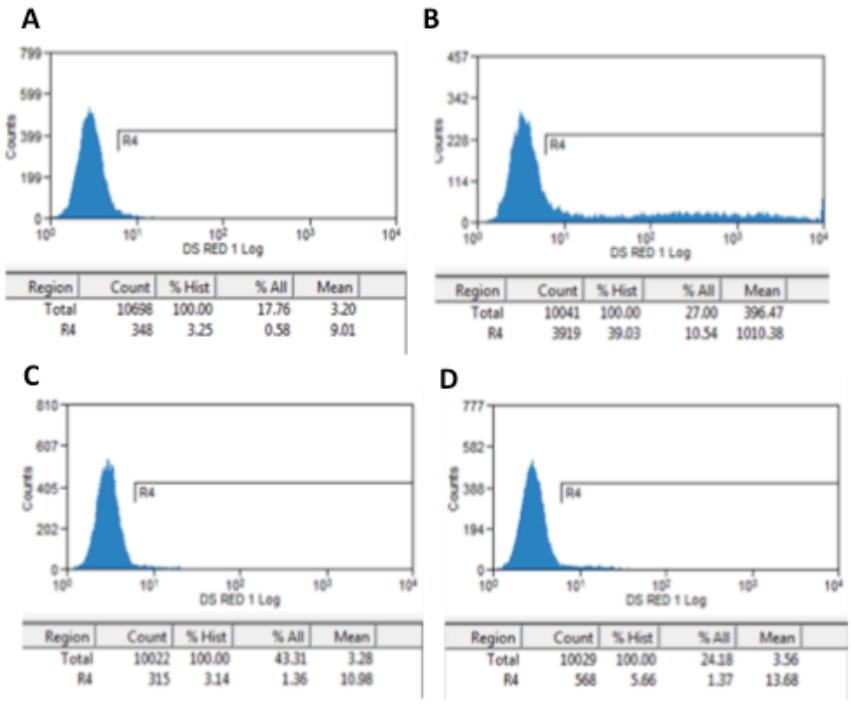


Figure 3.4 Comparison of PEI and 293Fectin transfection efficiency of HEK293F cells. HEK293F cells were transfected with pDNA coding for the DS red fluorescent protein (DS-RFP) as a reporter of transfection efficiency. The number of DS-RFP positive cells was compared between two transfection reagents, 293Fectin (LifeTechnologies) and PEI. Control conditions were exposed to transfection reagents alone (without DNA) whilst transfections were carried out according to manufacturers or published protocols for PEI (Durocher et al., 2002). A) HEK293F cells exposed to 293Fectin alone. B) HEK293F cells transfected with 293Fectin. C) HEK293F cells exposed to PEI alone. D) HEK293 cells transfected with PEI.

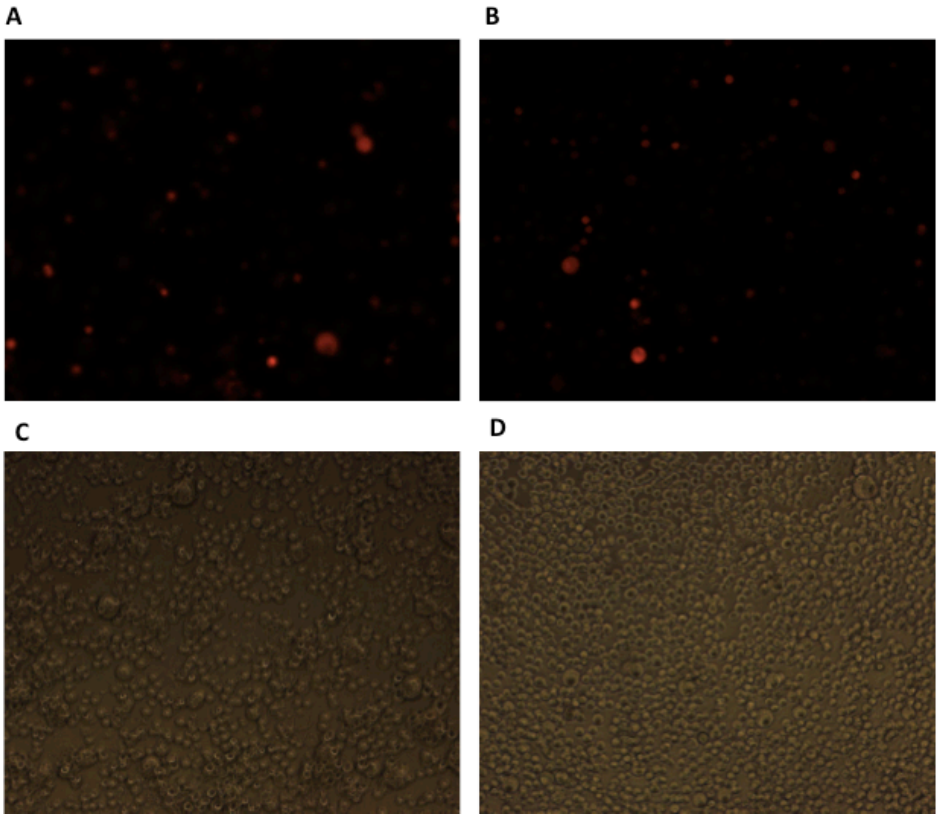


Figure 3.5 HEK293F cells transiently expressing red fluorescent protein 24 h after transfection. HEK293F cells were transfected with pDNA coding for the DS red fluorescent protein (DS-RFP) as a reporter of transfection efficiency after transfection with either PEI or 293Fectin. The suspension HEK293F cells were plated on microscope slides after 24 h for fluorescence and cell morphology analysis. Cell in images A and C were transfected with 293Fectin. Cell in images B and D were transfected with PEI. Changes in cell morphology were observed after transfection with 293Fectin when compared to control and PEI transfected cells. These changes included cell clumping and enlargement as can be seen in image C.

3.2.2.3 Optimisation of polyethylenimine to DNA ratio

Whilst manufacturer's guidelines are supplied with most commercial kits, published literature on optimum ratios of PEI to DNA for cell lines remains inconsistent. These factors have a significant effect not only on the transfection efficiency of cell cultures but also on recombinant protein expression. Compared to 293Fectin, PEI transfection efficiency was both lower and more variable (cells positive for the reporter protein were between 5.66-24%). Hence, the transfection protocol was optimised using a range of DNA:PEI ratios selected from the literature (Durocher et al., 2002; Akinc et

al., 2005; Pham et al., 2006; Backliwal et al., 2008b; Codamo et al., 2011; Longo et al., 2013).

HEK293F cells were seeded at $0.5x10^6$ cells/mL, in 30mL of Freestyle serum-free expression medium 24 h prior to transfection, to obtain a culture in log growth phase. Table 3.1 shows the DNA:PEI ratios investigated during the study and resulting GFP positive cells (%) in transfected populations 48 h later.

DNA (µg/MVC)	PEI (µg/MVC)	Cells positive for GFP (%)
0.5	1	23.55
1	1	5.66
1	2	38.34
1	4	11.34
1	8	2.58

Table 3.1 Effect of DNA: PEI ratio on percentage of GFP expressing HEK293 cells in 30mL suspension culture. HEK293F cells at a density of $1x10^6$ cells/mL were transfected with plasmid DNA coding for GFP according to the above PEI:DNA ratios. The percentage of cells positive for GFP expression was analysed 48 h after incubation using flow cytometry as described previously. A DNA to PEI ratio of 1:2 µg/ million viable cells (MVC) yielded the greatest percentage of GFP positive cells (38.34%).

The results showed that a DNA:PEI ratio of 1:2 resulted in an improved transfection efficiency of over 30% compared to the worst performing ratios. These conditions were used for transfection of expression plasmids. Having established the optimum transfection ratios these parameters were used in future experiments and scale up of production.

3.2.3 Establish the effects of transfection scale-up on cell growth, productivity and protein stability

Further characterisation of SM3EL-Fc, including *in vitro* and *in vivo* experiments, demanded increased production capacity from small shake flask based expression. Scale-up of transient transfection is often used as the simplest method for obtaining increased yields of recombinant protein (Derouazi et al., 2004). However scaling a transfection process does not necessarily maintain the same expression and growth characteristics as small-scale transfections.

In this experiment HEK293F cells were transfected as above using a directly scaled transfection approach with 10fold increases in cell culture volume (30mL, 300mL shake flaks and a 3L bioreactor) and subsequent increases in transfection solutions. At 30mL and 300mL scales the effect of PEI and 293Fectin was compared in shake flasks, however only PEI was used to transfect at 3L bioreactor scale due to cost limitations. The effect of transfection on cell growth characteristics was analysed immediately after addition of DNA complexes and over the successive culture period.

3.2.3.1 Viability of transiently transfected HEK293F cells

In preliminary experiments cytotoxicity was observed after exposure to both PEI and 293Fectin, hence transfections were carried out alongside control conditions where no DNA was included in order to establish the effect of the two reagents on cell viability without the increased metabolic stress of expressing protein.

Results showed that cells in 30mL and 300mL shake flasks subjected to either transfection reagent declined in viability at a greater rate than cells with no addition. Both reagents had a cytotoxic effect on cells within 24 h both with and without DNA included. However, the effect was less pronounced in cultures with PEI compared to 293Fectin. Viability of transfected cells decreased more than mock-transfected cells in both PEI and 293Fectin conditions. There was no effect of DNA addition alone on cell density or viability when compared to control cells. The DNA only condition was therefore eliminated from further experiments.

Scaling to the 300mL shake flask condition maintained similar viability profiles to 30mL cultures without such a dramatic reduction in viability after day 5-6. Again,

exposure to transfection reagents reduced cell viability compared no addition controls. 293Fectin reduced viability more than PEI in all shake flask conditions.

Scale up of transfection to 3L bioreactor fermentation showed comparable results to shake flask cultures with a drop in viability after transfection as observed at smaller scales. Bioreactor based cultures recovered from transfection at a faster rate than those grown and transfected in shake flasks but produced a lower total cell density of only $2.8x10^6$ cells/mL; substantially less than in shake flasks.

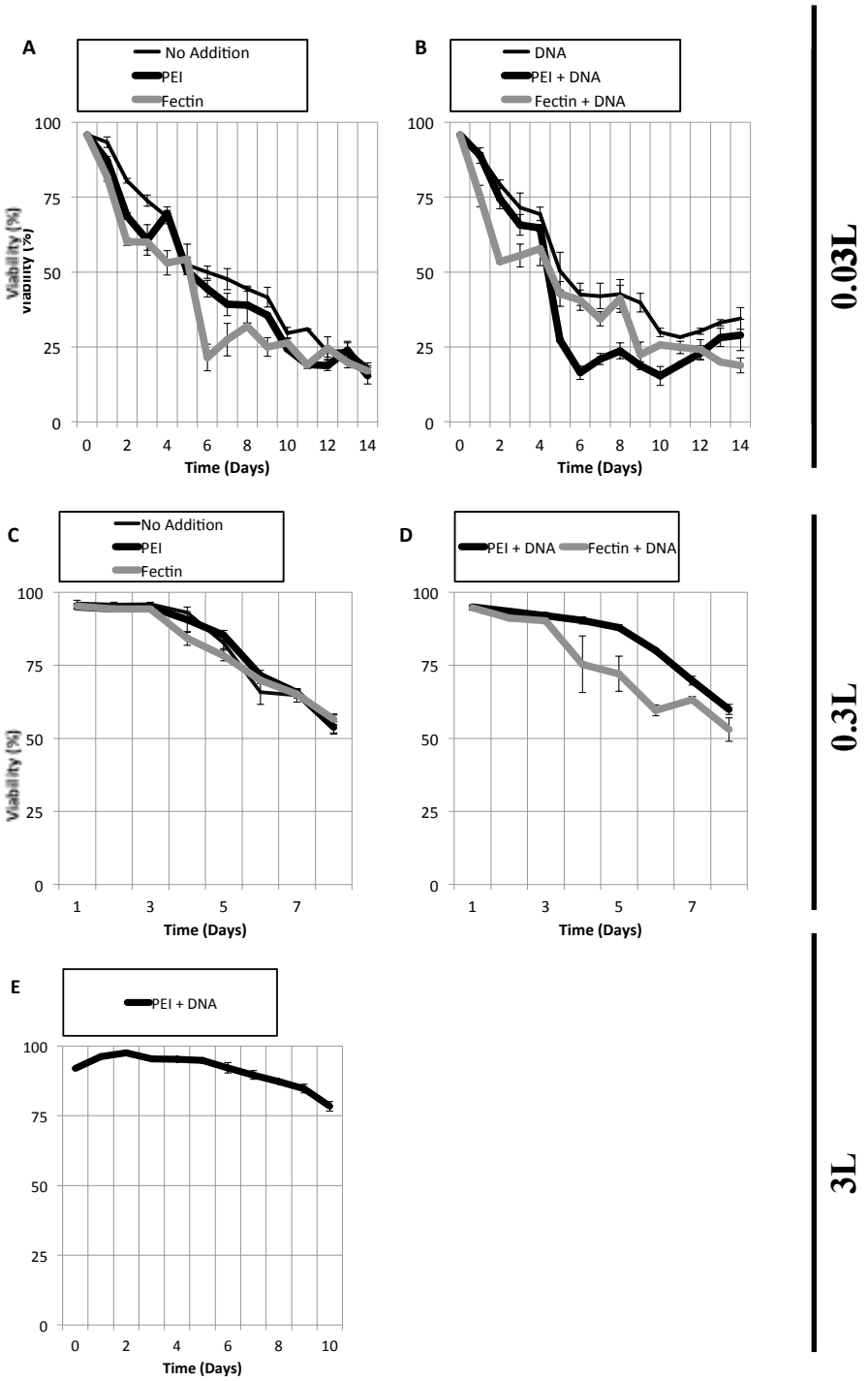


Figure 3.6 Viability of HEK39F cell cultures after PEI and 293Fectin transient transfection. HEK293F cells cultures were transfected at 30mL, 300mL and 3L scales using either PEI according to the previously optimised protocol or 293Fectin according to the manufacturer's instructions. Viability was determined via the Trypan blue exclusion assay using a Vi-Cell XR automated cell counter (Beckman Coulter, High Wycombe, UK). A) 30mL cultures with no DNA addition. B) 30mL cultures with DNA addition. C) 300mL cultures with no DNA addition D) 300mL cultures with DNA addition. E) 3L bioreactor culture transfected with PEI:DNA complex only. Error bars represent 1 standard deviation away from the mean (n=3).

3.2.3.2 Growth of transiently transfected HEK293F cells

Exposure of 30mL scale cultures to transfection reagents reduced total cell number achieved over the culture period compared no addition controls. Again, 293Fectin had a greater impact than PEI on total cell growth (Figure 3.7). There was also a marked reduction in total cell numbers when DNA was included in PEI mediated transfection complexes, supporting previous results. However, the same effect however was not observed in the 293Fectin transfected cells.

Upon scale up to 300mL there was an increase in the total cell density of transfected conditions of almost double when compared to the 30mL transfections. The cell density of transfected and mock-transfected cells was not significantly reduced. In the 3L bioreactor transfection, a low cell growth rate was observed after transfection compared to shake flask growth. The reactor culture reached a maximum of $2.67x10^6$ cells/mL and began to reduce in density after a shorter growth period.

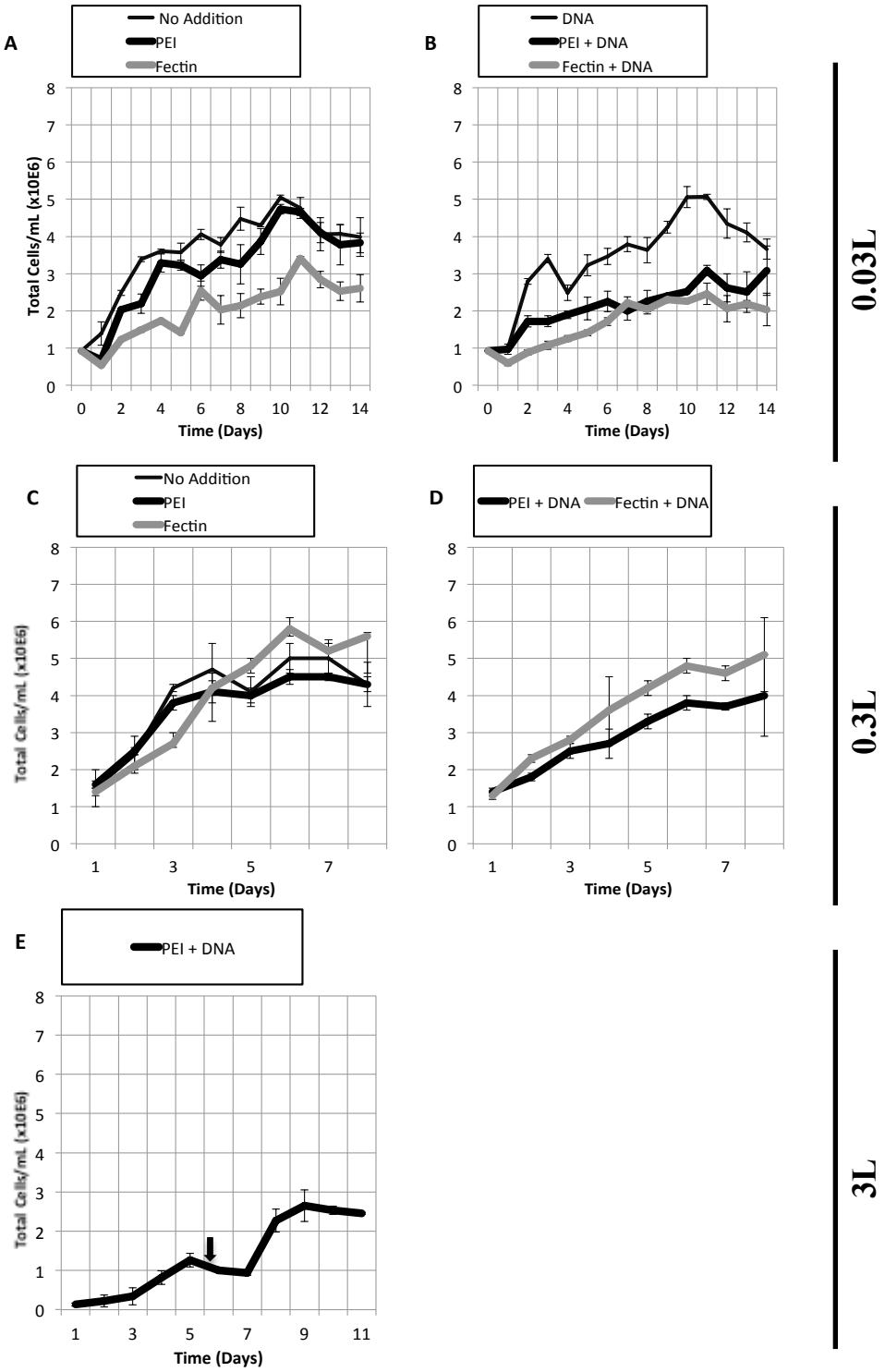


Figure 3.7 Total cell growth HEK39F cell cultures after PEI and 293Fectin transient transfection. HEK293F cells cultures were transfected at 30mL, 300mL and 3L scales using either PEI according to the previously optimised protocol or 293Fectin according to the manufacturer's instructions. Cell density was determined using a Vi-Cell XR automated cell counter. A) 30mL cultures with no DNA addition. B) 30mL cultures with DNA addition. C) 300mL cultures with no DNA addition D) 300mL cultures with DNA addition. E) 3L bioreactor culture transfected with PEI:DNA complex only, arrow indicates transfection point. Error bars represent 1 standard deviation away from the mean (n=3).

3.2.3.3 The effect of transfection scale on scFv-Fc expression

Secretion of SM3EL-Fc facilitates simple quantification in the supernatant using a specific plate-trapped antigen enzyme linked immunosorbant assay (PTA-ELISA). ScFv-Fc titres were analysed by removing samples daily, separating solids and storing the supernatant at -80°C to ensure accurate analyses of sFv-Fc content.

Although transfection efficiency was greater with 293Fectin (3.2.2.2), there was an increase of ~48% in scFv-Fc expression with PEI (60μg/mL and 38μg/mL respectively) at 30mL. Unsurprisingly, there was no expression of scFv-Fc detected in cells given DNA only. When scaled to 300mL there was an increase in the productivity over time as well as maximal scFv-Fc in the supernatant (PEI maximal - 72μg/mL and 293Fectin maximal expression of 61μg/mL) (Figure 3.8). In the 3L bioreactor however, expression seemed to follow the expression profile of 30mL cultures with a stable period of around 3 days of high expression followed by a rapid decrease.

In all conditions secreted scFv-Fc reached slightly less than the maximal level for each culture around 24 h (Figure 3.8) after transfection. This result correlates with the predicted cell cycle period for HEK293F cells in exponential growth, a prerequisite for the transfection process to take place.

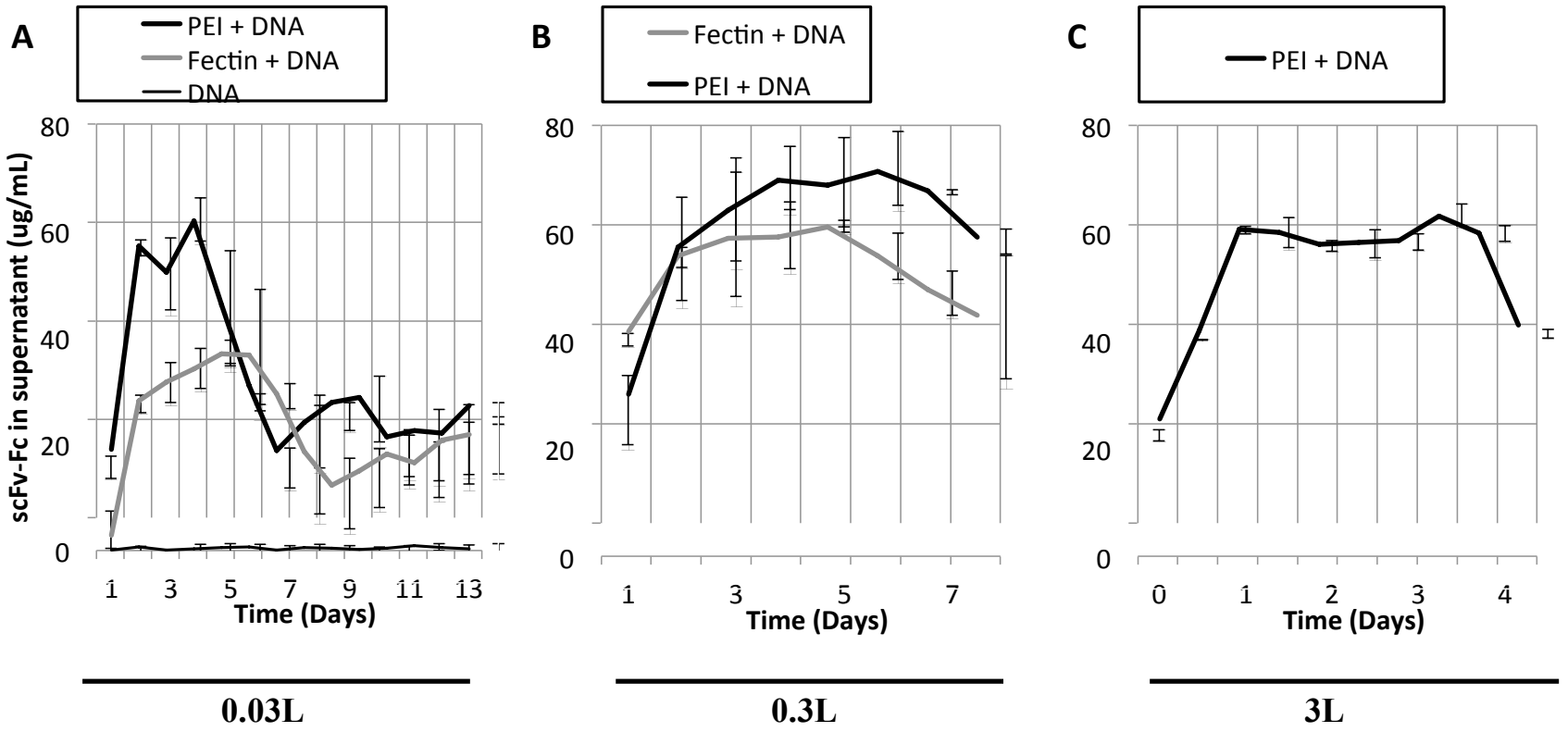


Figure 3.8 Total SM3EL-Fc in supernatant of transiently transfected HEK293F cells. HEK293F cells cultures were transfected at 30mL, 300mL and 3L scales using either PEI according to the previously optimised protocol or 293Fectin according to the manufacturer's instructions. Samples were removed daily from cultures of each scale, centrifuged and the supernatant stored at -80°C until required. The supernatant was assayed for scFv-Fc content using a target specific PTA-ELISA. A) 30mL shake flask. B) 300mL shake flask. C) 3L pneumatic SUT bioreactor. Error bars represent 1 standard deviation away from the mean (n=3).

3.2.3.4 The effect of transfection scale on scFv-Fc stability

SM3EL-Fc was purified from shake flask and bioreactor cultures using protein A MabSelect chromatography (2.10.1). The purified protein from the different scales and transfection methods was assessed and compared by SDS-PAGE after the culture period (Figure 3.9). There was no difference in the breakdown of protein harvested from cells transfected with the different transfection reagents at each scale, even though the total numbers of cells, and therefore potential for protease activity, differed substantially.

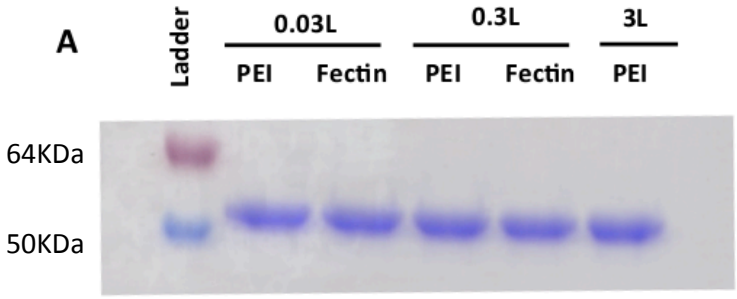


Figure 3.9 SDS-PAGE separation of transiently expressed SM3EL-Fc. Purified scFv-Fc resulting from transient expression at three different scales (30mL, 300mL and 3L) was diluted to 1mg/mL prior to addition of reducing buffer (see **Table 2.2**) and incubation at 100°c for 5 min. The proteins were resolved on a 10% SDS-PAGE gel and the number and size of bands compared between samples. There was no discernible difference between the products coming from the different scales.

Analysis of temperature dependant protein stability was carried out via differential scanning fluorimetry (DSF). Proteins were incubated with SYPRO Orange, a fluorescent dye that binds to exposed hydrophobic protein regions, and subjected to a temperature ramp of 1°C per 15 seconds. As temperature increases the amount of unfolded protein also increases enabling greater binding of SYPRO orange to hydrophobic residues. The fluorescence of bound SYPRO was analysed after every degree increase to obtain an analysis of key temperature transitions and stability characteristics. Key transitions analysed include the onset of denaturation, peak fluorescence and the temperature at which 50% of the protein is denatured, the Tm50. The Tm50 can be calculated via a first derivative curve analysis of the raw

data and is most often used to compare the thermal stability of proteins (**Figure 3.**10).

As expected the DSF results showed that there was no difference between the T_{m50} of scFv-Fc from cells transfected with PEI or 293Fectin (Tm50 =66.5°C and 66°c respectively) or between production scales (Figure 3.11).

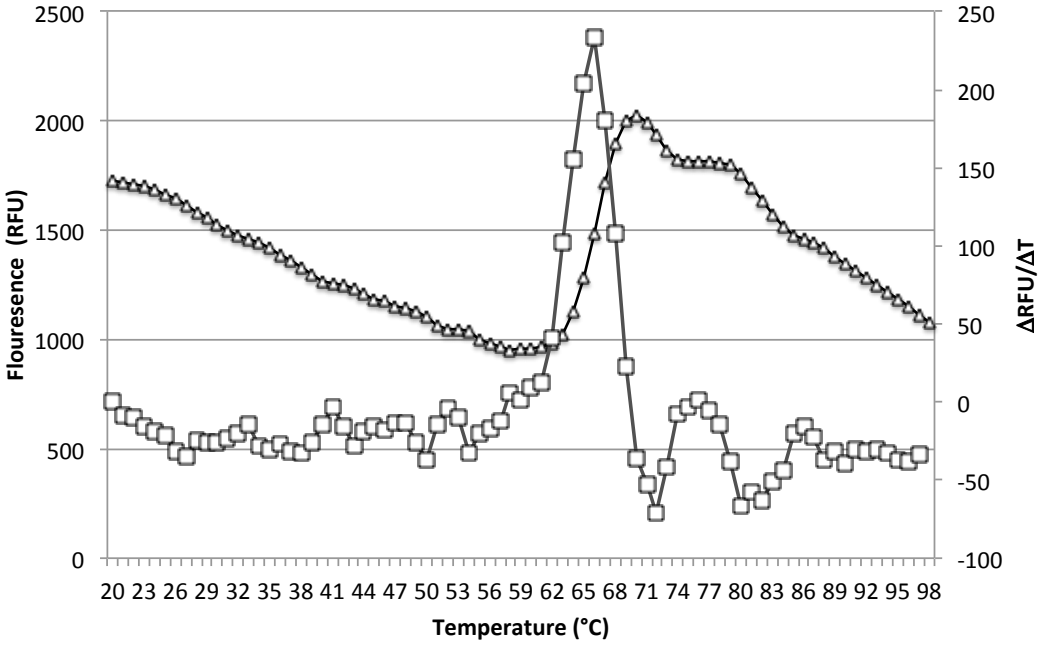


Figure 3.10 Temperature dependant stability of SM3EL-Fc from 30mL HEK293F cell transfection. SYPRO orange is a fluorophore that binds to hydrophobic protein regions allowing analysis of protein unfolding via differential scanning fluorimetry. SYPRO orange was diluted to a 1x concentration in 25µl of purified scFv -Fc (1mg/mL) in PBS buffer. Temperature dependant protein unfolding was assessed by heating from 20°C to 99°C at a rate of 1°C/15 s followed by a fluorescence read using excitation and emission of 480nm/568nm. The temperature of equilibrium between folded and unfolded protein (Tm50) was established using a first derivative curve analysis. This data shows Triangles indicate SYPRO fluorescence (RFU), squares indicate plot of first derivative function (ΔRFU/ΔT).

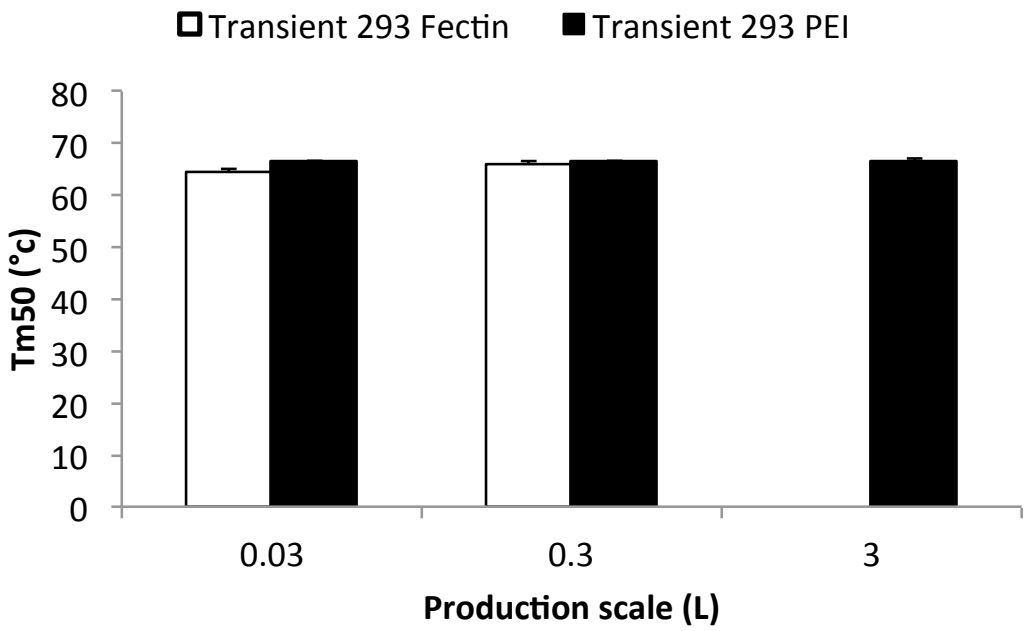


Figure 3.11 Comparison of temperature dependant stability of SM3EL-Fc produced at three different scales using two different transfection reagents. Differential scanning fluorimetry was used to analyse temperature dependant protein unfolding of scFv-Fc from the range of production scales and transfection reagents used in previous experiments. These include the reagents PEI and 293Fectin at scales 30mL, 300mL and 3L (PEI only) transfections. There was no difference between the Tm50 values for the proteins. Error bars represent 1 standard deviation distance from the mean (n=3).

3.2.4 Characterise the affinity and *in vitro* binding of scFv-Fc to the target antigen

3.2.4.1 Determination of the affinity of SM3E-Fc to NA1 via association and disassociation curve fitting

Kinetic association (K_a) and disassociation (K_d) constants of SM3EL-Fc were determined via surface plasmon resonance using a T200 enabled Biacore machine. A diagram depicting the assay designs used here can be seen in Figure 3.12. Equilibrium dissociation constants were established using this data according to Equation 4. The binding kinetics of SM3EL-Fc for NA1, the specific epitope of CEA to which it is targeted, were analysed using recombinant NA1 (Tolner et al., 2013). A literature review of SPR analysis protocols for antibody-antigen interactions recommended immobilisation of the antibody fragment with the ligand (antigen) passed over it, however during early stage experiments the amount of NA1 available proved a limiting factor in experimental design. Therefore, the epitope (NA1) was immobilised for SPR analysis rather than the scFv-Fc conjugate.

$$K_D = \frac{k_d}{k_a} = \frac{(\mathrm{A}b)(Ag)}{(AbAg)}$$

Equation 4 Equilibrium dissociation constant (K_D). A dissociation constant describes the relationship between bound and unbound antibody and antigen; where *Ab* is antibody and *Ag* is antigen. k_a represents kinetic association (K_{on}) and k_d represents kinetic dissociation curves (k_{off}).

3.2.4.2 Immobilisation of the NA1 epitope of CEA to Streptavidin chip

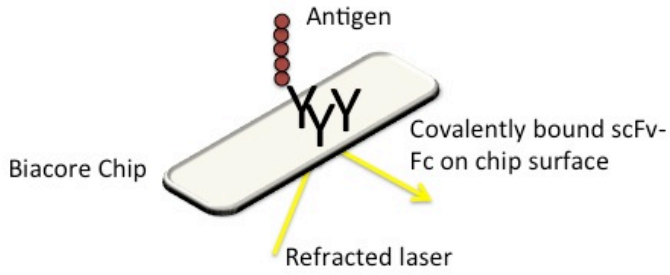
NA1 was covalently biotinylated using an EZ-Link Sulpho-NHS-LC-Biotinylation kit from Invitrogen (see methods) and desalted using a PD-10 column before immobilisation on a pre-prepared streptavidin coated (SA) Biacore chip from GE Healthcare (Buckinghamshire, UK) using the manufacturer's protocol. A 10nM solution of NA1 was prepared and immobilised at 800RU (Figure 3.13) in cell 2 of the chip, leaving cell 1 blank as a negative control.

3.2.4.3 Kinetic affinity analysis of SM3E-Fc on NA1

SM3E-Fc was applied to the NA1-SA-chip at concentrations of 400, 200, 100, 50, 25 and 12.5nM with subsequent regeneration of the chip carried out with 10mM glycine

at pH 2. Results indicated that there was excellent binding compared to negative control cells giving KD results that exceeded the detection limitations of the Biacore. Therefore only estimated reports could be generated, giving a KD (M) of 3.818×10^{-12} (low picomolar range affinity) (Table 3.2 and Figure 3.14). Whilst these results indicated an exceptionally high affinity of SM3EL-Fc for NA1 the experiment was repeated using immobilised scFv-Fc in order to achieve more accurate results.

A.



B.

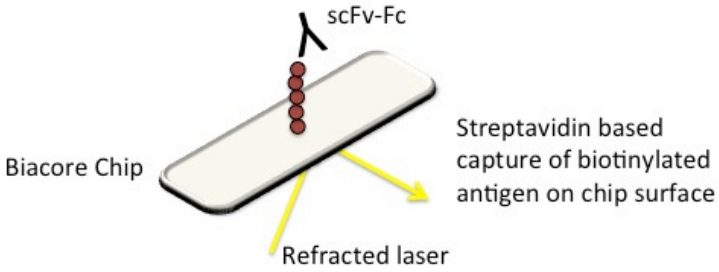
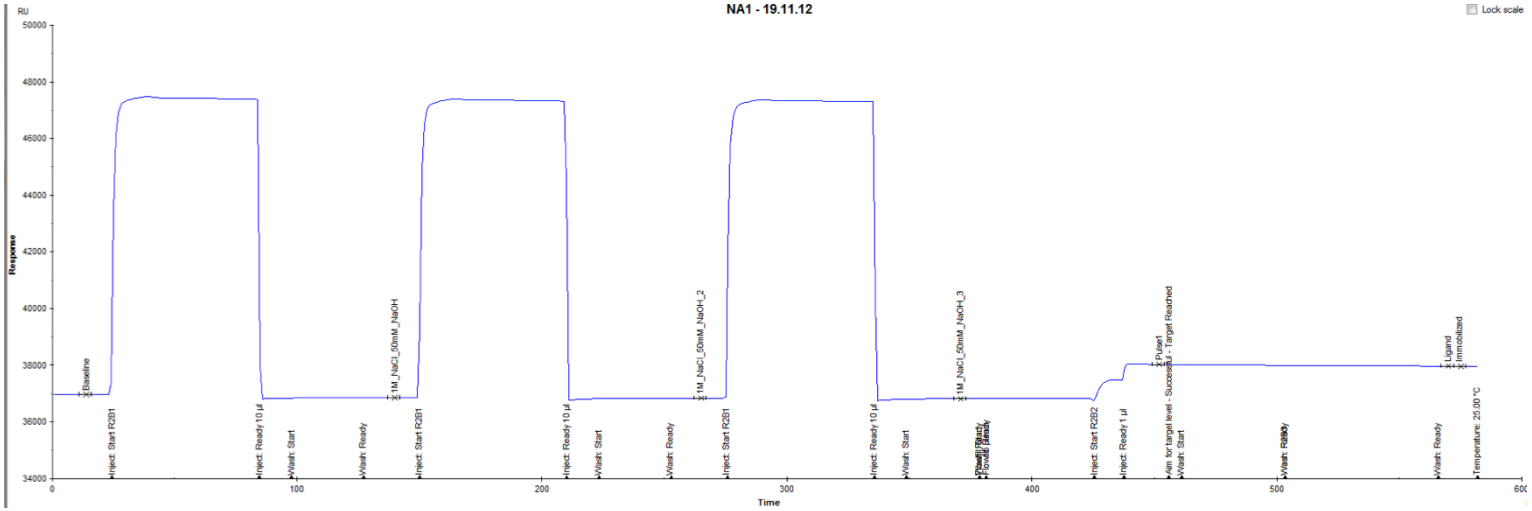


Figure 3.12 Diagram of typical surface plasmon resonance (Biacore) chip assay set up. An immobilised ligand (in this instance either the scFv-Fc (A) or the antigen (B)) is attached to the surface of the chip prior to measurement of binding of an analyte. Capture of proteins on the chip surface can be achieved using a range of methods, most commonly amine coupling (covalent binding), His-tag to Ni-NTA binding or biotin-avadin binding. Changes in laser-angle refraction are measured in relative fluorescence units (RFU) for binding analysis.



Fc	Time	Window	AbsResp	SD	LRSD	Slope	RelResp	Baseline	Id
3	14.0	5	36957.2	0.02	0.01	0.01	0.0	Yes	Baseline
3	140.0	5	36860.7	0.43	0.00	0.23	-96.5	No	1M_NaCl_50mM_NaOH
3	265.0	5	36836.5	0.53	0.02	0.28	-120.7	No	1M_NaCl_50mM_NaOH_2
3	371.0	5	36815.0	0.73	0.21	0.38	-142.1	No	1M_NaCl_50mM_NaOH_3
3	452.0	5	38026.8	1.57	0.06	-0.84	1069.7	No	Pulse1
3	570.0	5	37967.0	0.86	0.04	-0.46	1009.8	No	Ligand
3	575.0	5	37964.9	0.76	0.01	-0.41	1007.7	No	Immobilized

Figure 3.13 SPR sensorgram showing immobilisation of biotinylated NA1 on a streptavidin chip. The NA1 region of CEA was expressed and purified from *Pichia pastoris*. Immobilisation of NA1 for affinity analysis was carried out by biotinylation of the protein and binding to a streptavidin coated Biacore chip. Biotinylated NA1 was de-salted using a PD10 column (GE Healthcare) and immobilised on the chip at ~1000 relative response units prior to affinity analyses via surface plasmon resonance.

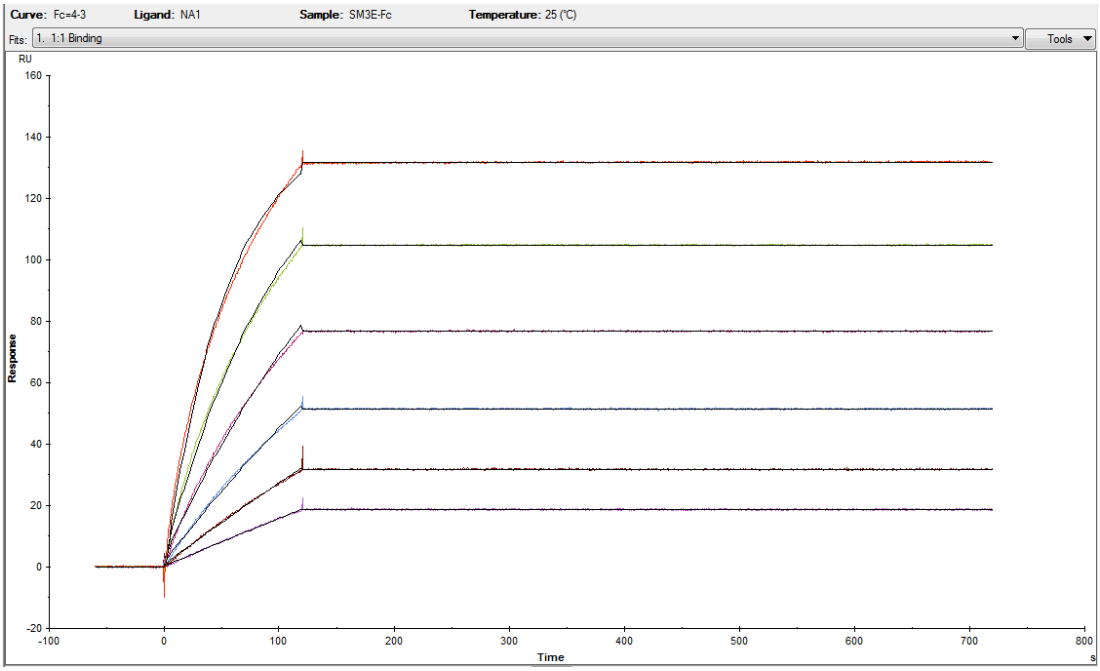


Figure 3.14 Sensorgram showing SM3EL-Fc binding to immobilised NA1. Purified SM3EL-Fc was passed over immobilised NA1 at concentrations of 400nM (red), 200nM (green), 100nM (pink), 50nM (blue), 25nM (brown) and 12.5nM (purple) in HEPES-P buffer (see **Table 2.3**). Association (k_{on}) and dissociation (k_{off}) phases occurred over 120s and 300s respectively and the chip was regenerated using 10mM Glycine-HCL, pH2.5. Binding kinetics and affinity constants were obtained using the BIAEvaluate software.

Quality Control | Report | Residuals | Parameters

Curve	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Conc (M)	tc	Flow (ul/min)	kt (RU/Ms)	RI (RU)	Chi² (RU²)	U-value
	4.623E+4	1.765E-7	3.818E-12			7.952E+8				0.374	95
Cycle: 24 12.5 nM				280.4	1.250E-8		20.00	2.159E+9	0.09438		
Cycle: 25 25 nM				246.3	2.500E-8		20.00	2.159E+9	0.5616		
Cycle: 26 50 nM				213.1	5.000E-8		20.00	2.159E+9	1.380		
Cycle: 27 100 nM				180.6	1.000E-7		20.00	2.159E+9	2.451		
Cycle: 28 200 nM				156.4	2.000E-7		20.00	2.159E+9	2.090		
Cycle: 29 400 nM				147.7	4.000E-7		20.00	2.159E+9	-3.320		

Table 3.2 Affinity (K_D) of SM3EL-Fc for immobilised NA1 antigen. Purified SM3EL-Fc was passed over immobilised NA1 at concentrations of 400nM, 200nM, 100nM, 50nM, 25nM and 12.5nM in HEPES-P buffer (see **Table 2.3**). The K_D was estimated to be 3.818×10^{-12}M affinity.

3.2.4.4 Immobilisation of SM3EL-Fc to CM5 chip via EDC/NHS coupling

Immobilisation of SM3EL-Fc was carried out using amine-coupling chemistry on a Biacore CM5 Amine chip according to the manufacturer's instructions (see 2.16). However this method was unsuccessful under standard binding conditions. Binding buffer for the immobilisation was optimised between parameters of pH 4-5.5 in 10mM Acetate (Figure 3.15). Results indicated that the binding response varied substantially depending on pH. A maximum relative response of 32695RU was possible at pH 4.5 compared to a minimum of 1150RU at pH 5.5. Subsequent binding cycle analysis confirmed results from pH scouting with a maximum relative response of 32540RU in cycle 3 correlating to pH 4.5 (Figure 3.16).

EDC/NHS activation of the CM5 amine chip was carried out prior to SM3EL-Fc addition in 10mM acetate (pH4.5) binding buffer. Final immobilisation of SM3EL-Fc was reduced to 442.9 RU to ensure accuracy of kinetic analysis. As described previously a reference cell (1/4) was left blank on the chip (3.2.4.2). The same immobilisation technique was carried out with two different scFv-Fc constructs, one with affinity for NA1 and one without. Quality control of the chip was achieved by flowing a 200nM solution of NA1 over all cells of the chip. The results showed positive binding in the two cells with scFv-Fc's that had affinity for NA1 and none in either the blank cell or the cell immobilised with a non-binding scFv-Fc (Figure 3.17, Figure 3.18 and Table 3.3).

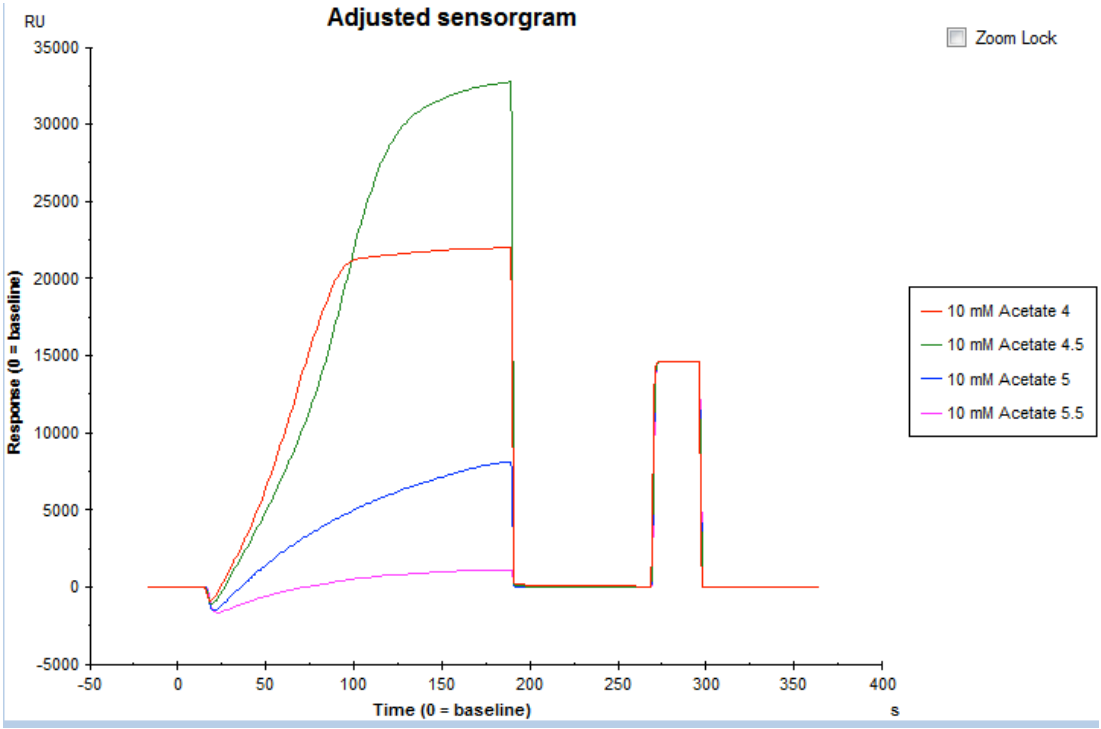


Figure 3.15 Sensorgram showing relative response of SM3EL-Fc in binding buffers of different pH. Binding conditions for the immobilisation of SM3EL-Fc using amine-coupling were optimised using binding buffers of 10mM acetate from pH 4 to 5.5. The relative binding response of the scFv-Fc in each buffer was measured to ensure the optimum buffers for immobilisation were used. Association phases of scFv-Fc in different buffers was analysed over 180s K_a rates were similar between buffers of pH 4 and 4.5 whilst lower binding was observed at pH5.5.

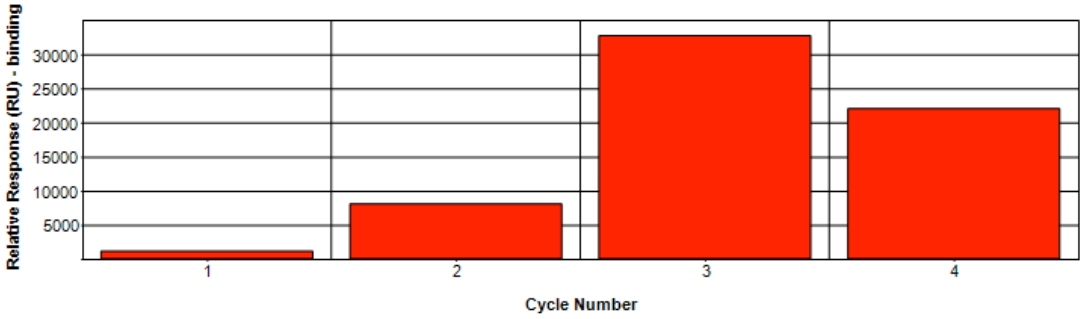


Figure 3.16 Maximum binding (RU) of SM3EL-Fc to CM5 chip pH altered buffer. Consecutive cycle numbers relate to binding buffer 1) 10mM Acetate pH5.5. 2) 10mM Acetate pH5.0. 3) 10mM Acetate pH4.5 4) 10mM Acetate pH4. Maximum relative response indicates that buffer composed of 10mM acetate pH 4.5 was optimal giving the highest RU of 32540 compared to the lowest of 1150RU at pH5.5.

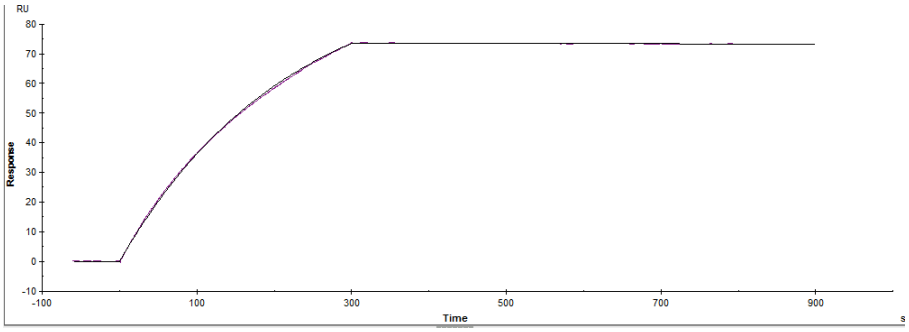


Figure 3.17 Chip testing with binding of NA1 to immobilised SM3EL-Fc. SM3EL-Fc was described earlier as an anti-CEA scFv-Fc, it was predicted to bind with high affinity to the NA1 ligand. The NA1 epitope was passed over the immobilised scFv-Fc as a concentration of 200nM confirming activity of the immobilised construct at ~72 RU.

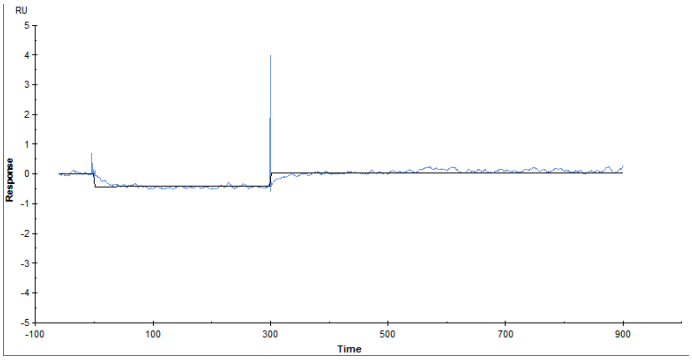


Figure 3.18 Chip testing with binding of NA1 to non-binding scFv-Fc. B6L-Fc is an scFv-Fc with no affinity for CEA/NA1. As predicted there was no binding of NA1 to B6L-Fc confirming that there was no non-specific binding activity of NA1 to the chip surface.

Immobilised Construct	Ka (1/Ms)	Kd (1/s)	KD (M)	Rmax (RU)	Chi^2
SM3EL-Fc (Anti-NA1)	$2.32x10^{4}$	$3.68x10^{-6}$	$1.586x10^{-10}$	97.81	0.0725
B6L-Fc	-	-	-	-	-

Table 3.3 Binding of NA1 solution to immobilised scFv-Fc. SM3EL-Fc was immobilised on a CM5 amine Biacore chip (GE Healthcare). A 200nM solution of NA1 was passed over the chip with

3.2.4.5 Kinetic affinity analysis of NA1 on immobilised SM3EL-Fc

The affinity of SM3EL-Fc to NA1 again resulted in the system being capable of calculating only a predictive K_D, however this was no longer in the picomolar range dropping to $1.58x10^{-10}$. Having analysed the affinity of the scFv-Fc for the target when immobilised to a Biacore chip and passed over an NA1 immobilised chip, the predictive value had varied in terms of overall K_D. In order to clarify the result and confirm binding to the whole molecule in a more biologically relevant fashion the assay was repeated using the whole CEA molecule.

3.2.4.6 Kinetic affinity analysis of CEA on immobilised SM3EL-Fc

CEA has previously proved a difficult protein to use in SPR analysis, possibly due to it's heavily glycosylated structure, hence the use of the NA1 epitope in previous experiments. However for a further affinity analysis of SM3EL-Fc, CEA binding was tested using commercially available CEA from R&D. The same SM3EL-Fc immobilised chip was used for analysis of CEA binding and NA1 after regeneration. Results showed that the affinity of SM3EL-Fc for the whole CEA molecule was predicted to be in the same range as the NA1 region alone with a KD of $1.881x10^{-12}$ compared to $3.818x10^{-12}$ on immobilised NA1. The correlation between these results increased confidence in the results suggesting that the affinity of SM3EL-Fc was in the picomolar range (Figure 3.19).

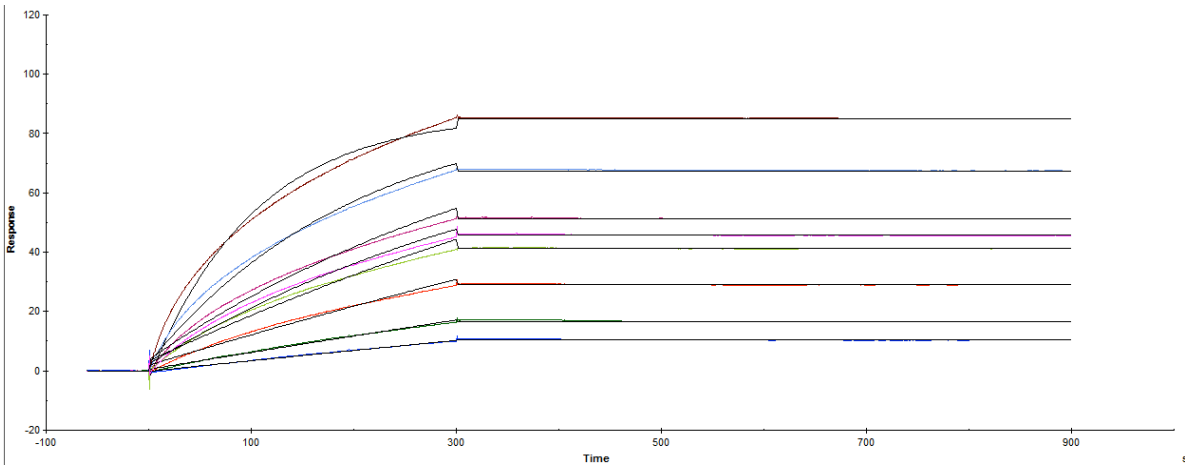


Figure 3.19 Sensorgram showing binding of CEA to immobilised SM3EL-Fc. Recombinant CEA was passed over the chip at 200nM (red), 100nM (blue), 50nM (pink x2), 25nM (green), 12.5nM (red), 6.125(dark green) and 3.125nM (dark blue) in HEPES-P buffer (see section 2.16). Association (k_{on}) and dissociation (k_{off}) phases occurred over 300s each and the chip was regenerated using 10mM Glycine-HCL, pH2.5. Binding kinetics and affinity constants were obtained using the BIAevaluate software.

Curve	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Conc (M)	tc	Flow (ul/min)	kt (RU/Ms)	RI (RU)	Chi² (RU²)	U-value
	4.880E+4	9.178E-8	1.881E-12			1.111E+9				0.965	95
Cycle: 4 200 nM				89.64	2.000E-7		30.00	3.451E+9	-3.081		
Cycle: 5 100 nM				87.61	1.000E-7		30.00	3.451E+9	2.478		
Cycle: 6 50 nM				98.53	5.000E-8		30.00	3.451E+9	3.601		
Cycle: 7 25 nM				134.3	2.500E-8		30.00	3.451E+9	3.150		
Cycle: 8 12.5 nM				173.7	1.250E-8		30.00	3.451E+9	1.778		
Cycle: 9 6.25 nM				190.7	6.250E-9		30.00	3.451E+9	0.4052		
Cycle: 10 3.125 nM				233.0	3.125E-9		30.00	3.451E+9	-0.1900		
Cycle: 12 50 nM				87.83	5.000E-8		30.00	3.451E+9	2.117		

Table 3.4 Binding kinetics of immobilised SM3EL-Fc for recombinant CEA. Recombinant CEA was passed over the scFv-Fc immobilised to the chip at a concentrations of 200nM, 100nM, 50nM, 25nM, 12.5nM, 6.125 and 3.125nM. An estimated K_D of 1.881×10^{-12}M was calculated using k_a and k_d values with the BIAevaluate software.

3.2.4.7 I*n vitro* localisation of SM3EL-Fc in CEA expressing tissue

Having assessed the binding of SM3EL-Fc to recombinant CEA, the *in vitro* targeting of SM3EL-Fc to CEA in the natural conformation was analysed by localisation to the target on Capan-1 cells at 4°C for 1 h (Figure 3.20). Bound SM3EL-Fc was stained with anti-murine IgG conjugated alexafluor 488 whilst the nucleus was stained with DAPI. Results showed a green florescence signal to be evident around the surface of the cell in the expected location of GPI-linked CEA whilst there was no comparative staining to on the CEA negative A375 cell line (Figure 3.21). *In vitro* localisation of SM3EL-Fc corroborated previous results and confirmed successful binding of the protein to CEA in its natural conformation on the cell surface with showed no off target activity.

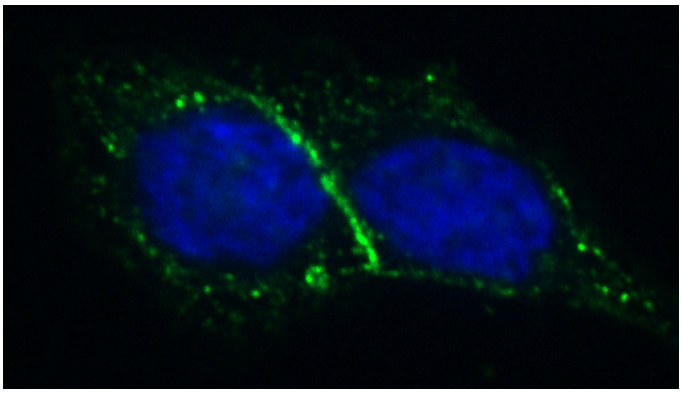


Figure 3.20 Localisation of SM3EL-Fc on CEA expressing Capan-1 cells after 1 h. SM3EL-Fc was added to Capan-1 cells and incubated at 4°C for 1 h. Secondary staining of the scFv-Fc was achieved using an anti-murine IgG conjugated Alexafluor 488 (green) whilst the nucleus is stained using DAPI (blue). Image with help of Dr Enrique Miranda Rota.

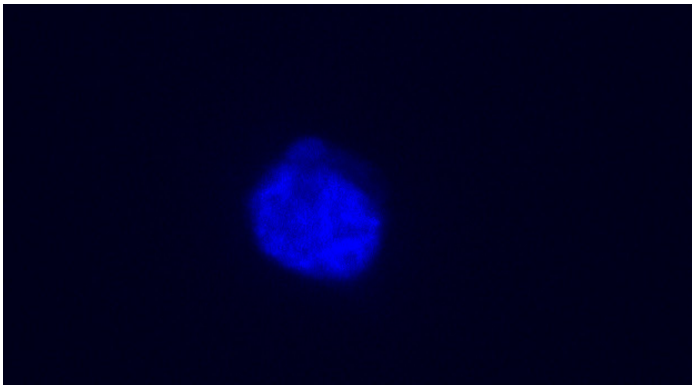


Figure 3.21 Localisation of SM3EL-Fc on A375 cell (CEA negative cell line). SM3EL-Fc was added to a CEA negative cell line (A375) and incubated for 1 h at 4°C. Secondary staining of the scFv-Fc was achieved using an anti-murine IgG conjugated Alexafluor 488 (green) whilst the nucleus is stained using DAPI (blue). Image with help of Dr Enrique Miranda Rota.

3.3 Discussion

The aims of this chapter were to develop a mammalian expression system that worked in a rapid, scalable and cost effective fashion allowing production of recombinant proteins at lab scale. Many protein expression systems have substantially improved yields and lower costs, however mammalian cells have the advantage of being able to produce correctly glycosylated proteins that do not course immune responses due to post-translational modifications (Zhu, 2011) and retain functional characteristics. Despite this the cost and time of mammalian cell based protein expression can remain a barrier in some instances.

TGE has a number of advantages over traditional stable cell based gene expression including no requirement for selection of stably integrated cells and thus no need to use reporter genes allowing for significantly reduced time between transfection to purification (Durocher et al., 2002). In practise this means that essential work on often-novel proteins can begin quickly allowing characterisation, without attributing time and costs to potentially unproductive stable gene selections. In-turn, this has led to its increased popularity, especially at lab scale (Geisse and Henke, 2005).

CHO cells are the predominant mammalian system for recombinant protein expression usually in the form of stably transformed cells. Whilst HEK293 cells have advantages over CHO for TGE of r-proteins (Thomas and Smart, 2005) it would be advantages to use the final expression system for preliminary protein production in order to establish potential faults with the protein early rather than after stable cell selection and protein characterisation. For this reason both CHO and HEK293F cells were investigated as potential expression systems for the scFv-Fc.

CHO cells yielded low quantities of protein after transient transfection, a maximum of 3.84μg/mL, despite high numbers of viable cells and rapid growth whilst HEK293F cells expressed ~8.6 μg/mL. Analysis of the transfection efficiency of suspension CHO and HEK293F cells via GFP/RFP expression showed that the efficiency of CHO cell transfection with PEI was substantially lower than that of HEK293F cells (<2% and ~39% respectively) after optimisation. As a result the HEK293F system yielded a maximum yield of 72 μg scFv-Fc /mL meaning it was selected as the

preferred expression system. Transfection of HEK293 cells can produce in the range of 50mg/L- 1g/L (Geisse and Henke, 2005; Baldi et al., 2007; Liu et al., 2008). TGE expression can be improved using a number of techniques are available to increase protein yield from TGE including the use of valproic acid (Backliwal et al., 2008c), cell cycle inhibitors (Tait et al., 2004) and apoptotic inhibition (Majors et al., 2008), however the range of expression yields present in the literature are indicative of the high variation in expression of even proteins of similar sequence (Pybus et al., 2014b).

Transfection reagents range hugely in price and productivity. Hence, a comparison of two different transfection reagents, PEI and 293Fectin, was carried out during scale up to investigate differences in the productivity, cell growth and stability of the resulting protein. PEI is a simple to make solution that has been used as a lipofection platform in many well cited studies (Backliwal et al., 2008b; Benjaminsen et al., 2013; Codamo et al., 2011; Durocher et al., 2002; Longo et al., 2013) costing in region of £70/g, meanwhile 293Fectin (Life Technologies) has a price of £405/mL.

The 293Fectin reagent outperformed PEI in terms of transfection efficiency as determined by RFP expression however also had greater cytotoxic effects on cells. The results shown here indicate that PEI out performed the more expensive reagent in terms of productivity. It should be noted that all there is greater potential for batch-to-batch variation in the use of PEI solutions, as they are generally made in house.

Direct scale up of the transfections to 300mL flasks produced an improved yield compared to small shake flasks using both reagents (PEI=72μg/mL and 293Fectin = 61μg/mL). The hypothesis that improved maintenance of culture conditions in the bioreactor would produce an improved protein yield was not accepted after further scale up to a 3L bioreactor using PEI transfection produced a similar yield to the 30mL shake flask condition. Whilst transfection appeared to reduce the total cell growth of cell cultures in the 30m and 3L conditions, the effect was less pronounced in the 300mL flasks potentially explaining the increase in protein yield.

Analysis of protein stability was carried out as part of the product characterisation and to compare the stability of proteins resulting from different scales and reagents. The

scale effects of transfection have not been fully characterised, hence there was potential for protein stability to be affected by cell growth and lysis (Hutchinson et al., 2006). Results from differential scanning fluorimetry showed that the average Tm_{50} of SM3EL-Fc was 66.5°C and that there was no difference between the stability characteristics of proteins from the different scales and reagents analysed. Obtaining comparative stability data for other scFv-Fc constructs has proved difficult, (4 thesis examples in the range of 55-64°C) however when the assay was applied to whole antibodies the results conformed within 0.2-3°C of published results suggesting that the assay was accurate (King et al., 2011; Niesen et al., 2007). These data were corroborated by SDS-PAGE analysis of the proteins showing that there was no difference in the breakdown profile of the product after purification.

Increased stability of antibodies and scFv-Fc constructs is often associated with improved affinities (Fennell et al., 2013). Dissociation constants of scFv-Fc constructs are usually in the nM range (Hashiguchi et al., 2003; Rochefort et al., 2014), however the predicted affinity of the scFv-Fc discussed here is in the 10^{-12} M (picomolar) region for both the whole CEA molecule and the specific epitope of the antigen. Whilst high affinity of constructs for targets does not always correlate with increased therapeutic efficacy in the context of solid tumour therapy (Adams et al., 2001; Rudnick et al., 2011; Vasalou et al., 2015), these results suggested that the scFv-Fc would be an effective binder.

To assess localisation of SM3EL-Fc to its target in a biological context and test potential non-specific interactions of the scFv-Fc *in vitro* binding assays were completed. *In vitro* binding of SM3EL-Fc to CEA expressing cells was carried out on capan-1 cells with a375 cells used as a negative control. The results showed good localisation of the scFv-Fc to the target protein and no binding to the CEA negative cell line. These results confirmed the binding of the scFv-Fc to the target in its natural conformation and showed there were no non-specific interactions. The scFv-Fc was therefore validated as a functional model for use in further expression experiments.

3.3.1 Conclusions

The aims of this chapter were to develop a cost effective transient expression system to produce quantities of recombinant protein sufficient for pre-clinical characterisation. These aims were achieved by establishing the HEK293F cell line along with PEI based transfection as a suitable expression platform that could be scaled up to 3L. The lower cost transfection reagent was shown to be as effective as the more expensive reagent after optimisation of the transfection methods.

Analysis of the model protein produced using multiple scales and reagents showed that there was no difference in product quality. SPR affinity analysis and *in vitro* binding showed that the scFv-Fc bound to its target antigen specifically and with affinity predicted to be in the low picomolar range.

The results in this chapter show that significant quantities of protein can successfully be produced by transient expression at multiple scales and analysed to short time scales using inexpensive reagents and techniques.

Chapter 4 Stable expression of SM3EL-Fc in a CHO cell population; yield and quality comparison with transiently expressed protein

4.1 Introduction

The work in this chapter focuses on the use of stable integration of transgenes to increase recombinant protein production capabilities without the need for clonal selection. Whilst TGE can reliably and rapidly produce sufficient amounts of protein for early protein characterisation studies, it faces a number of limiting factors for increased protein manufacture. This includes the need to re-transfect cells after each batch, synthesis of large amounts of recombinant DNA for scale up and potential for heterogeneity between batches. HEK293F cells have demonstrated improved transfection efficiency and productivity over CHO cells during TGE, however the literature points to CHO cells providing greater recombinant protein yields during stable expression thanks to increased cell densities of up to $1.5x10^7$ cells/mL and the use of expression enhancing technology (Kim et al., 2011).

Whilst the initial protocols for transient and stable-based transfection of cells for gene expression are the same, stable gene expression relies on serendipitous integration of transgenes into the host cell genome prior to selection. The integration of exogenous DNA sequences occurs randomly within the genome of the host. The nuclear translocated plasmid DNA molecules (in either linear or circular form) are integrated into the host cell genome in nonhomologous recombination events during cell division (Finn et al., 1989). These recombination events can occur at multiple loci and with erratic frequency. Multiple copies of the transgene are often present at the same locus due to ligation of numerous copies of plasmid DNA to form a concatamer prior to recombination (Perucho et al., 1980).

The heterogeneous protein expression and cell characteristics exhibited by transfected cell populations can there be apportioned to the lack of control over the locus or dosage of the transgene to the genome. During stable cell selection, these factors play an important role in determining the expression profile of the protein. Cells with the highest gene expression are usually selected based on resistance to antibiotic or metabolic stress. These highest expressers often have the transgene integrated into

highly expressed genomic areas such as those linked to 'house keeping' genes and may also have multiple transgene copies (Zhang et al., 2010).

Rapid generation of high expressing cell populations is of particular relevance to therapeutic recombinant protein manufacture as stable cell line generation and selection of high expressing clones can take as long as 6 months. The ability to produce high expressing cell populations removes the need for clonal isolation and screening to obtain the best expressers, removing a significant step in cell line development. In this chapter, two methods for improving simple, rapid generation of stable cells were explored; controlled locus of transgene integration and incorporation of a ubiquitous chromatin opening element (UCOE) based expression vector to enhance gene expression and reduce silencing.

Transgene integration can be engineered to occur at specific recombination using a two-stage integration and selection process employing a DNA recombinase (Flp recombinase) derived from *Saccharomyces cerevisiae* that carries out site-specific recombination at previously defined binding and cleavage sites as shown in Figure 4.5. When the number and locus of flp cloning sites in a cell line is known to be conducive to high expression, as assayed by a reporter gene, transfection can generate a high yielding population of cells quickly. Random integration of the scFv-Fc, SM3EL-Fc into Hela wild type cells was carried out in order to compare productivity with flp-mediated transfection.

The second method explored in this chapter is the enhancer-less ubiquitous chromatin-opening element (UCOE). In contrast to flp recombinase mediated transgene integration, the UCOE construct impacts directly on genomic structure at the locus of integration by almost completely stopping DNA methylation of the integrated transgene and generating a substantial increase in gene expression (Zhang et al., 2010). Whilst this technique is based on random integration, it generates high expressing cell lines in a single step using strong promoter activity and reduces gene silencing by regulation of heterochromatin structure via the heterogeneous nuclear ribonucleoproteins A2 B1/chromobox protein homolog 3 (HNRPA2B1-CBX3) house keeping gene locus (Williams et al., 2005).

This chapter aims to investigate methods to generate high expressing stable cell pools that do not require clonal selection for early production purposes. This was achieved using the model scFv-Fc described previously and either flp-targeted transgene recombination or UCOE based expression vectors. Both techniques aim to generate a high proportion of good expressers resulting in a more rapid cell line development process for early production needs. Increased production capacity was utilised for further work including assessment of *in vivo* and *in vitro* efficacy and distribution. This chapter also compares proteins from transient and stable cell systems in terms of stability and effector function to explore differences in scFv-Fcs produced in the different expression systems.

Aims

Investigate methods to generate stable cell pools for high expression of recombinant protein and to compare scFv-Fc produced in stable and transient systems

Chapter objectives

- Establish antibiotic selection conditions for Hela and flp site expressing H2Z cells (2.1.3) and generate randomly integrated scFv-Fc expressing populations.
- Construct isogenic flp integrated Hela cell line expressing SM3EL-Fc
- Construct stable CHO-S cell line expressing SM3EL-Fc in UCOE vector
- Develop media and feeding strategy for maximum protein expression in shake flasks culture
- Characterise and compare *in vitro* effector function and stability of transient and stably expressed scFv-Fc.

4.2 Results

4.2.1 Zeocin and Hygromycin titration for Hela/H2Z-Flp selection

The sensitivity of wild type Hela cells to zeocin and hygromycin was assessed in order to facilitate selection of stable cells. After transfection, the cells would be sensitive to either hygromycin or zeocin dependent on the locus of integration inserting the transgene after the ATG start signal (see Figure 4.5).

Cells were exposed to zeocin concentrations of 0, 50, 100, 250, 500 or 1000μg/mL aiming for a selection period of between 4-5 days allowing time for expression of resistance genes and recovery from cytotoxic effects of transfection (Figure 4.1). Results showed that all zeocin concentrations affected the viability of cells, with those above 250 μg/mL reducing viability to below 20% after just 2 days. Cells subjected to 50 and 100 μg/mL were respectively 20% and 22.5% viable after 5 days, therefore a selection concentration of 100 μg/mL was chosen for further work.

Hygromycin concentrations of 0, 100, 250, 500, 750 and 1000 μg/mL μg/mL were also titrated in order to establish the appropriate selection concentration (Figure 4.2). The results indicate that a concentration of 250 μg/mL was sufficient to reduce cell viability of control cells to ≤ 20% at the desired time point of 5 days.

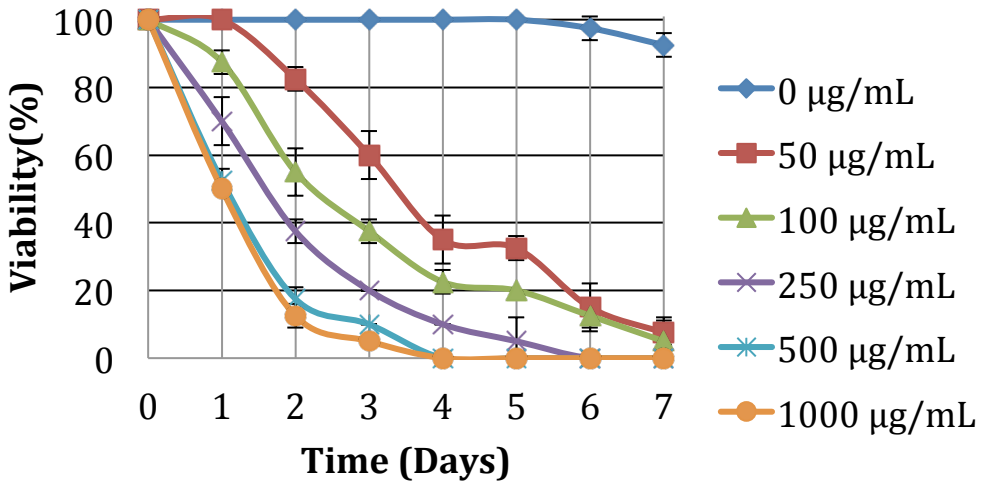


Figure 4.1 Titration of zeocin concentration required for selection of stable Hela cells. Zeocin selection was necessary as part of the integration of FRT sites into Hela cells. The cell lines was cultured to 70% confluence in 6–well plates and subjected to zeocin concentrations of 0, 50, 100, 250, 500 and 1000 µg/mL to establish selection concentrations for use in further experiments. A selection concentration of 100 µg/mL was chosen as the viability of Hela cells tested reduced to 20% over 4 days. Graph shows mean data and error bars show one standard deviation from the mean (n=3).

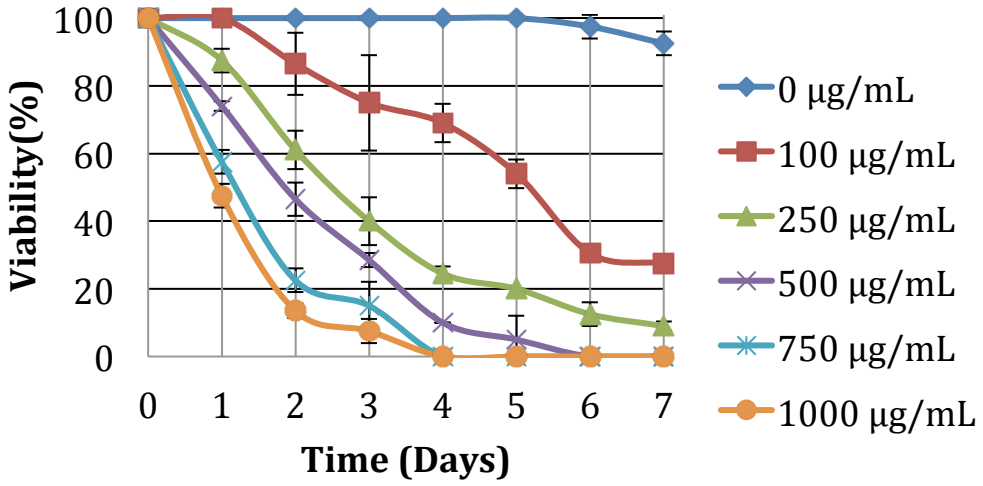


Figure 4.2 Titration of hygromycin concentration required for selection of stable Hela cells. Hygromycin resistance was necessary for selection of flp-recombinase integrated cells. Hela cells were cultured to 70% in 6-well plates and subjected to hygromycin concentrations of 0, 100, 250, 500, 750 and 1000 µg/mL to establish the selection concentrations for use in further experiments. A selection concentration of 250 µg/mL was chosen in order to obtain a viability of 20% over a period of 4 days. Graph shows mean data and error bars show 1 standard deviation from the mean (n=3).

4.2.2 Random integration and selection of SM3EL-Fc into Hela cells

Random integration of SM3EL-Fc into Hela wild type cells was carried out in order to compare productivity with flp-mediated transfection. Transfection was performed in T25 tissue culture flasks using branched PEI (25 kDa) as described in 2.5. The pFUSE-SM3EL-Fc vector contains the gene *sh ble,* conferring resistance to zeocin exposure, hence transfected cells were selected for stable integration using 100 μg/mL zeocin as described above. After 7 days exposure to zeocin the viability of transfected cells had reduced to 63% whilst the control cell population was no longer viable (Figure 4.3). The transfected cells went on to recover to 100% viability after 12 days and were cryopreserved at low passage number.

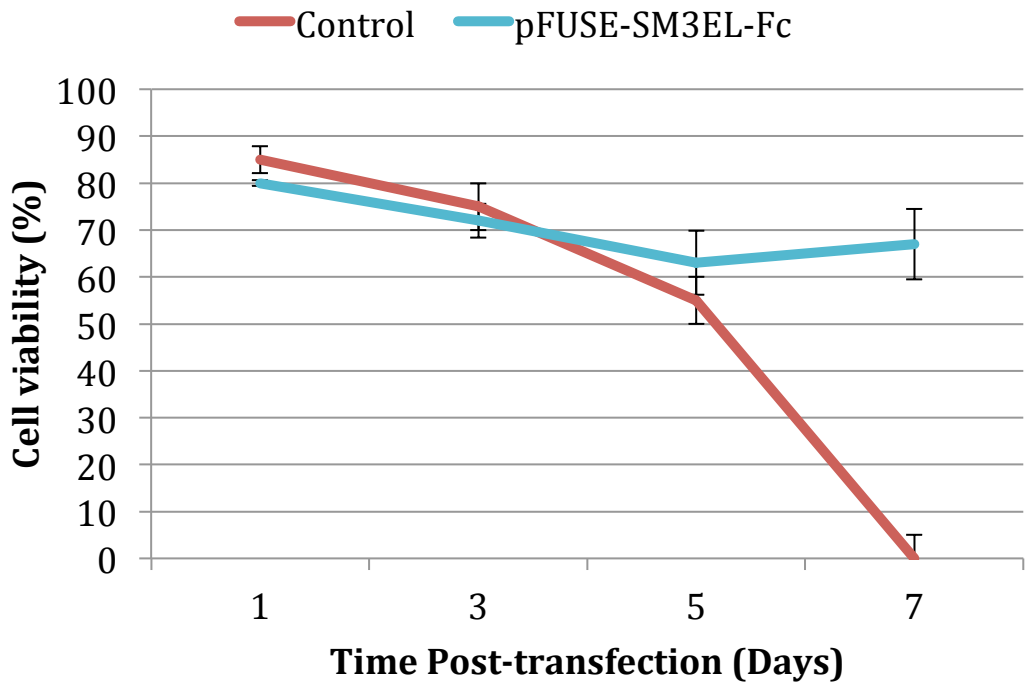


Figure 4.3 Selection of stable Hela cells randomly integrated with pFUSE-SM3EL-Fc. Adherent Hela cells were randomly transfected using branched form 25 kDa PEI in T75 flasks with transgenic plasmid DNA encoding the gene for the scFv-Fc as well as the *sh ble* gene conferring resistance to zeocin. After 24 h the cells were exposed to zeocin (100 µg/mL) and the viability determined by Trypan blue exclusion. Transfected cells (blue) remained over 60% viable whilst control cells (red) were 0% viable after day 7. Data shows mean viability and error bars indicated one standard deviation from it (n=3).

4.2.3 Expression analysis of three randomly selected Hela cell populations

Clonal selection of a number cell lines from the stable pool was carried out via single cell limited dilution in a 96-well tissue culture plate. Preliminary analysis of scFv-Fc expression by three clones showed the range of heterogeneity of randomly integrated Hela cells over the course of three weeks (Figure 4.4). After selection, cells were cultured in identical conditions (37°C, T75 flask, 5% CO2, seeded at $2x10^5$ cells/mL in DMEM media) and passaged at ~90% confluence. The supernatant was removed after each week and analysed for expression of SM3EL-Fc via NA1 specific PTA-ELISA (Methods 2.15.4).

There was no difference in the recovery of cells or growth rates of the three clones analysed, however the results indicated that there was a wide range in the expression

profiles even in the small sample number analysed. These results illustrate the expression heterogeneity in populations when transgene integration is carried out via random integration techniques.

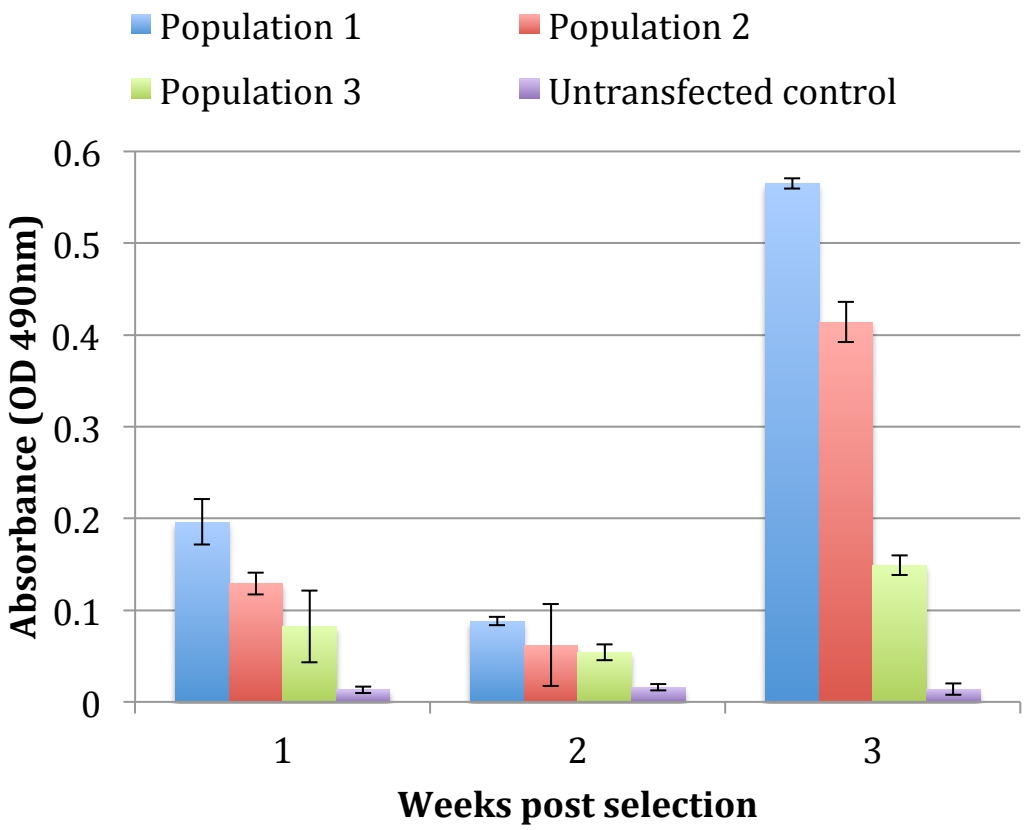


Figure 4.4 Randomly integrated Hela cell populations expressing SM3EL-Fc. Zeocin selected Hela cells stably expressing SM3EL-Fc were cultured in three separate populations after clonal selection to asses the heterogeneity between different populations. Cells were cultured until ~90% confluent before passaging and the supernatant was retained each week. Expression of the secreted scFv-Fc was analysed by ELISA. Protein concentration observed in the supernatants differed between time points as cells recovered from selection and between the three populations illustrating heterogeneity. Data shows mean viability and error bars indicated one standard deviation from it (n=3).

4.2.4 Construction of a Flp mediated isogenic cell population

The two-stage selection process for flp integration required cloning of DNA coding for SM3EL-Fc into a flp-site expression vector, transfection and selection procedures for cell integration and assessment of expression activity.

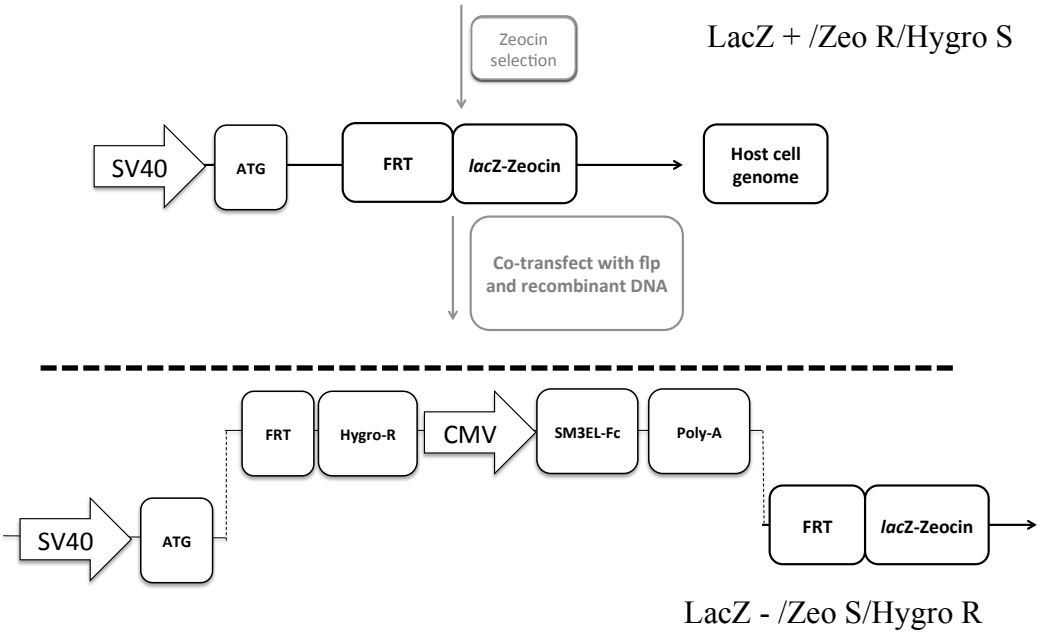


Figure 4.5 Two stage flp recombinase meditated integration of SM3EL-Fc into Hela cells for isogenic expression. Stage one - the FRT (flp recombinase targeting site) is integrated, via zeocin selection, into the host cell genome. Activity of the integrated sites and selection of clonal selection can be carried out according to βgalactosidase actvity. Stage two - co-transfection of DNA coding for flp recombinase and the protein of interest in the pcDNA5/FRT vector results in integration of the hygromycin resistance gene and the protein expression cassette and re-sensitises cells to zeocin. Accronyms; SV40 is the simian vacuolating virus 40 promoter, ATG is the start codon, FRT is DNA coding for the flp recombinase, lacZ sequence codes for β-galactosidase, Hygro-R is the hygromycin resistance gene, CMV is the cytomegalovirus promoter region, poly-A is the poly adenosine sequence and S signifies antibiotic sensitivity.

4.2.5 H2Z-Flp β-galactosidase activity validation

H2Z-Flp, a Hela cell line expressing flp recombinase targeting sites and the *lacZ* cassette (Figure 4.5, Stage 1), was kindly provided by Dr Darren Nesbeth, UCL. Cells were cultured in the presence of zeocin at a concentration of 100 μg/mL ensuring

antibiotic resistance was present. After 7 days of culture, β-galactosidase activity was analysed to confirm the presence of the promoter-linked flp-sites in cells.

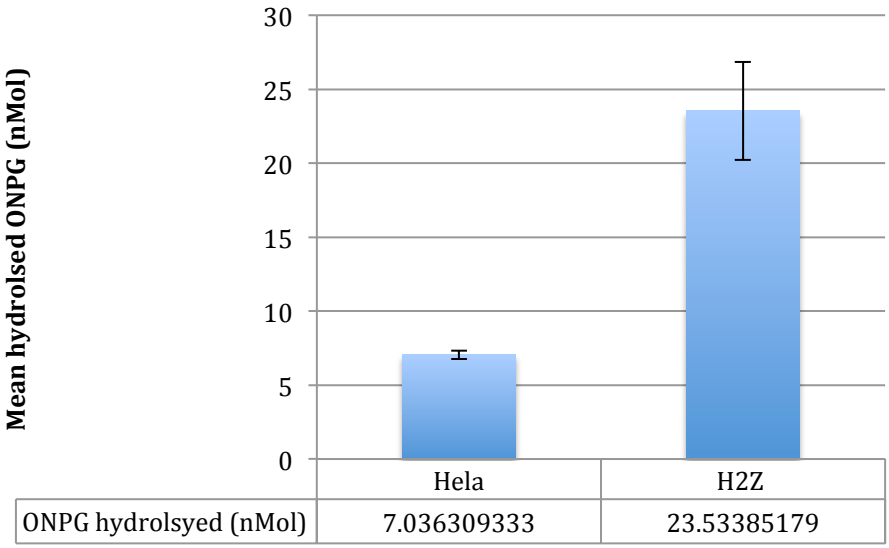


Figure 4.6 Confirmation of Beta-galactosidase activity by Hela cells with integrated FRT sites. Cells stably integrated with FRT-sites are also positive for β-galactosidase activity. Hence β-gal activity was assessed by hydrolysis of the substrate ortho-Nitrophenyl-β-galactoside (ONPG) by FRT integrated cells (H2Z) and compared to wild type Hela cells. Cells were harvested and pelleted before being lysed. The supernatant was recovered, ONPG added and the reaction incubated at 37°C for 30min allowing colour to develop. The assay was quantified using a microplate reader. Data represents the mean and error bars 1 standard deviation away from it (n=3).

β-galactosidase activity was analysed using the β-Gal Assay Kit from Life Technologies (UK). A colorimetric assay based on hydrolyses of the β-galactosidase substrate ortho-Nitrophenyl-β-galactoside (ONPG). Hydrolyses of ONPG results in galactose and the yellow coloured orthonitrophenol that can be assayed by absorbance of light. The rate of hydrolysis was compared between wild type Hela and H2Z-Flp cells. The results, as expected, showed that the H2Z-Flp cell line had a substantially greater rate of ONPG hydrolysis than the wild type (background) Hela cells (23.5 nM hydrolysed ONPG compared to 7nMol respectively) (Figure 4.6). These results demonstrated that β-galactosidase activity of the H2Z-Flp cells was present; hence the FRT site was also present due its position as part of the same transgene insert.

4.2.6 Cloning of SM3EL-Fc into Flp transfection vector for isogenic cell integration

Direct comparison of SM3EL-Fc expression between random and flp-mediated cell populations required use of identical protein expression cassettes (i.e. under the same promoter and control elements) in both conditions. To facilitate this the whole expression cassette for the protein, rather than just the scFv-Fc fragment, was cloned into the flp integration vector.Figure 4.7 shows a cloning strategy designed to achieve a direct comparison of expression. Restriction digest of the pFUSE-SM3EL-Fc expression fragment with NotI and BglI followed by electrophoresis and gel excision produced an insert of 3087bp after purification. The pCV01 vector encoding the flp recombination site (pCV01) was digested with SphI and HindIII, to remove the resident expression cassette including promoter and polyA region before also being gel excised to obtain a 4110bp backbone vector fragment. The vector and insert fragments were not complementary, therefore a blunt end ligation method was employed involving DNA polymerase I, large (Klenow) fragment blunting of both fragments, dephosphorylation of the vector with alkaline phosphatase (CIP – calf intestinal phosphatase) and phosphorylation of the insert via T4 polynucleotide kinase (PNK) (Figure 4.7).

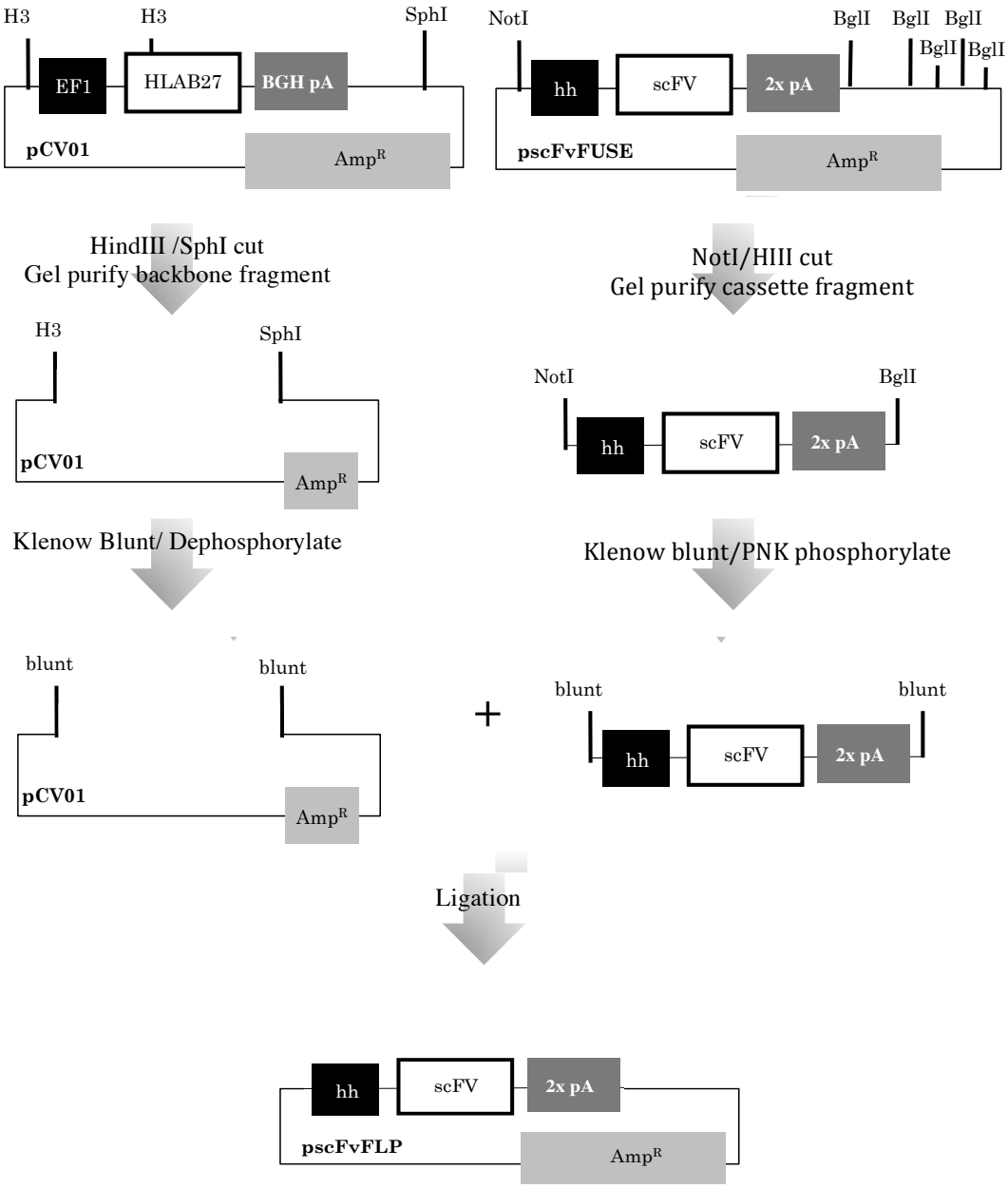


Figure 4.7 Cloning strategy for insertion of SM3EL-Fc expression cassette into-flp site encoding vector. The flp site-encoding vector (pCV01) was digested using the restriction enzymes HindIII and SphI to remove the resident expression cassette, promoter and polyA regions. The whole expression cassette of the scFv-Fc in the pFUSE vector was separated via restriction digest with NotI and BglI. Both the backbone and insert fragments were isolated using electrophoresis and gel excision. The purified fragments were blunted using DNA polymerase 1, large fragment (Klenow) to blunt the non-complimentary fragment ends. Prior to ligation the fragments were either phosphorylated or dephosphorylated using CIP (Calf Intestinal Phosphatase).

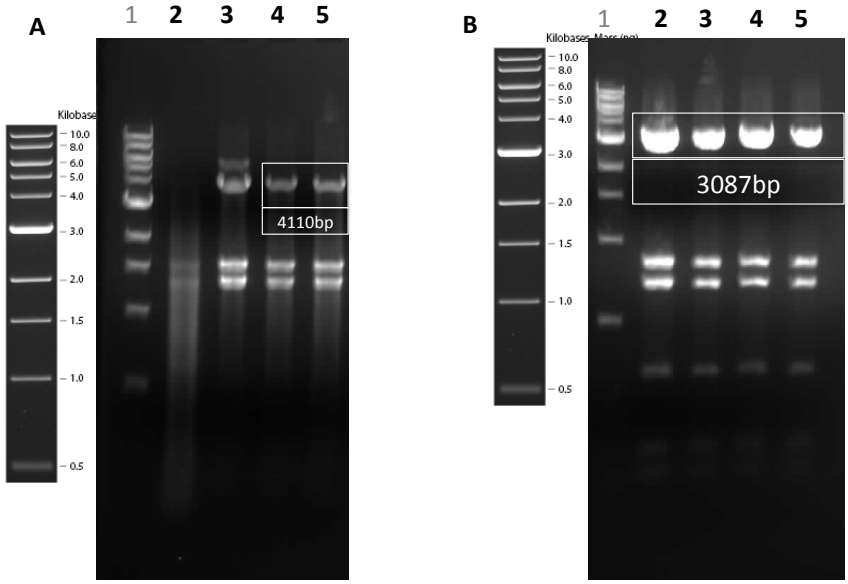


Figure 4.8 Resolution of restriction digested Flp vector and ScFv-Fc insert. pDNA was isolated from 4 different TG1 *E. coli* colonies. Restriction digest showed colonies with the expected digestion pattern and allowed gel excision of the desired fragment for cloning. A) The flp-encoding vector was digested using HindIII and SphI enzymes to produce a 4110bp backbone fragment vector (white box). B) pFUSE-SM3E-Fc was digested using NotI and BglI restriction enzymes to produce multiple fragments; the 3087bp fragment (white box) contains the whole expression cassette of SM3EL-Fc. The molecular weight marker in lane 1 of both images is the 1Kb ladder from NEB.

Subsequent ligation of insert to vector (Figure 4.8) along with appropriate controls was carried out and purified using phenol-chloroform extraction prior to transformation of electrocompetent *E. coli* Top10 cells. Transformed cells were plated out on 100 μg/mL ampicillin 2TY agar selection plates before overnight incubation at 37°C. The number of colony forming units was analysed to compare background vector re-ligation with ligated vector and insert as well as confirm ampicillin resistance in the uncut vector as positive control. The resulting colony ratios did not suggest that positive vector-insert ligations were present.

Multiple cloning attempts were carried out using this strategy including varying ligation reactions, vector:insert ratios, strains of *E. coli,* (de) phosphorylating fragments and PCR colony screening methods. This cloning strategy did not result in

cloned insert, however parallel cloning of SM3EL-Fc into a UCOE based expression vector had been successful therefore the decision was made to proceed with this system for expression of SM3EL-Fc in further experiments.

4.2.7 Cloning of SM3EL-Fc into UCOE vector

Sequence analysis of pFUSE-SM3EL-Fc showed that it lacked compatible restriction sites for cloning into the UCOE expression vector, hence the simplest method of cloning was to PCR amplify the murine SM3EL-Fc expression cassette and insert compatible (sticky end) restriction sites whilst maintaining the activity of the IL2 secretion signal. Primers were designed to extend the SM3EL-Fc cassette and insert the restriction sites 5'Bstb1 and 3' Xba1 (See Figure 4.9). The resulting 1543bp PCR fragment was gel extracted, purified and digested along with the vector using BstBI and XbaI restriction enzymes to create complementary ends. Ligation of the digested fragment into the 3.2Kb murine UCOE expression vector was carried out at room temp for 10 minutes before phenol-chloroform extraction. See Figure 4.10 for final plasmid construction map.

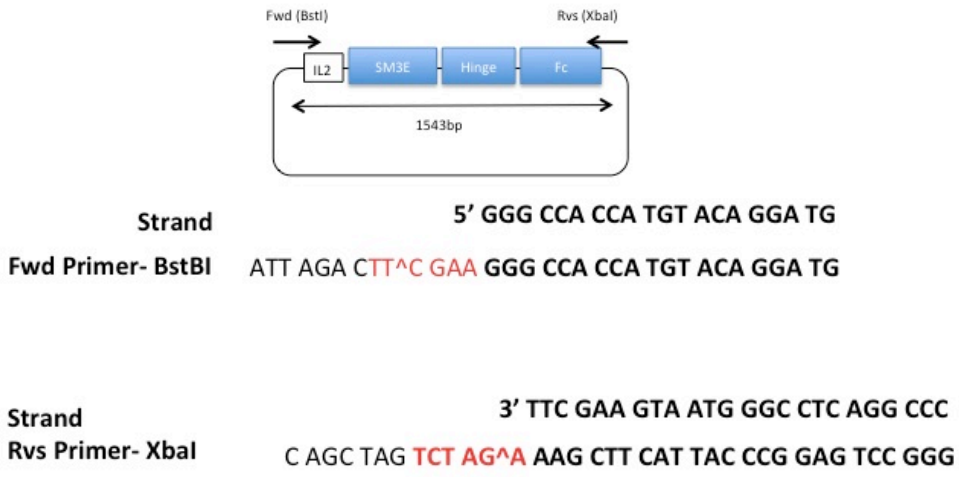


Figure 4.9 Extension primers for addition of BstBI and XbaI restriction sites to SM3EL-Fc for cloning into the UCOE vector. Cloning of SM3EL-Fc into the UCOE expression vector required extension of the expression cassette and addition of complimentary restriction sites for simple ligation into the MCS. The forward (sense) primer added the restriction site for BstBI whilst the reverse (anti-sense) primer added the XbaI site. Both restriction sites are indicated in the primers in red with '^' denoting the cutting site yielding an expected PCR amplicon of 1543bp.

The ligated plasmid was transformed via electroporation into Top10 *Escherichia coli*, plated on 100μg/mL ampicillin 2TY agar plates and incubated over night at 37°C. There were a greater number of colonies in the vector + insert conditions compared to control plates indicating increased likelihood of colonies positive for the ligated insert. Colonies were selected and grown in liquid 2TY media (Amp 100μg/mL). Plasmid DNA was recovered from 6 colonies and analysed via restriction digest with BglII. Clone 6 showed the correct digestion pattern (6670bp and 2632bp) (Figure 4.11). Sequencing (UCL sequencing service) was carried out using primers designed to provide data across the insert and Fc portion of the construct. This confirmed that no mutations had occurred during the cloning process

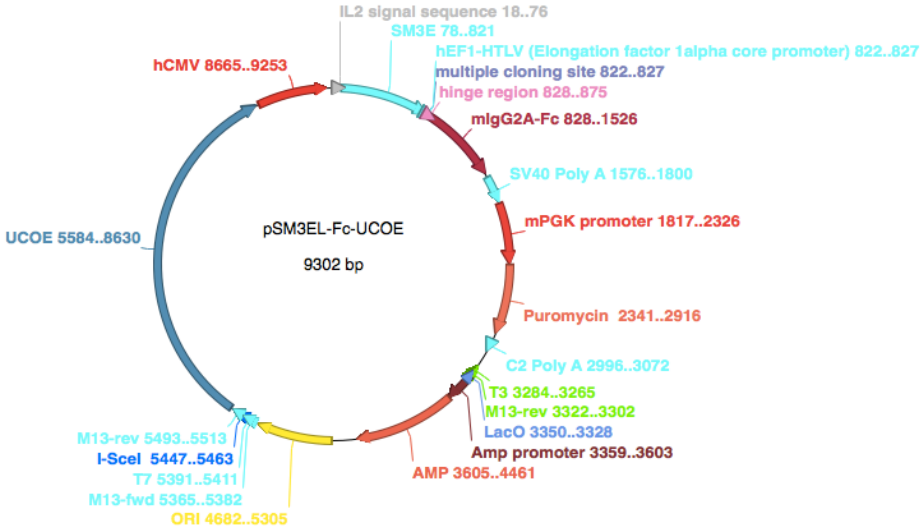


Figure 4.10 Plasmid map of pSM3EL-Fc-UCOE. The expression plasmid map shows the SM3E scFv (blue), hinge region (pink) and mouse Fc (red) under the control of the hCMV promoter and IL2 signal sequence as well as the ubiquitous chromatin-opening element (UCOE) that utilises house keeping promoter genes to reduce methylation based silencing and increase expression. The plasmid confers resistance to puromycin under the mPGK promoter for mammalian cell selection and ampicillin under the Amp promoter for bacterial selection purposes.

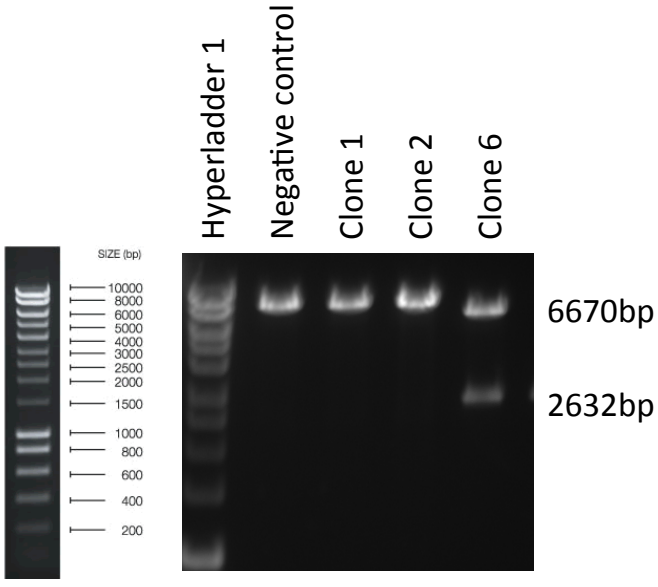


Figure 4.11 Restriction digest of pSM3EL-Fc-UCOE using BglII. Top10 *E. coli* were transformed with pSM3EL-Fc-UCOE via electroporation. Transformed cells were plated on 2TY-ampicillin agar plates and incubated overnight at 37°C. 10 CFUs were selected and transferred to liquid ampicillin selection 2TY media before pDNA was isolated and presence of the insert analysed. The pDNA was digested using BglII restriction enzyme. Clone 6 had the expected DNA fragment sizes of 6670bp and 232bp. Negative control was uncut DNA. The molecular weight marker was the Hyperladder 1 from Bioline.

4.2.8 Titration of puromycin for stable cell selection

The necessary concentration of puromycin to apply sufficient selection pressure for stable cell line generation was titrated prior to selections. Puromycin was added to untransfected CHO-S cells at a density of $1x10^6$ cells/mL at a concentration of 0, 5, 7.5, 10, 12.5, 15 and 20μg/mL (Figure 4.12). After 48 h of puromycin exposure cultures supplemented with either 10 or 12.5μg/mL reduced in viability dramatically to around 40% whilst those ≥15 μg/mL were below 5% viable. Cells with 5 μg/mL puromycin were unchanged compared to control whilst 7.5μg/mL showed a mild reduction in viability. In selection conditions of 7.5μg/mL and above there was a distinct morphological change in a proportion of the population, developing non-circular profiles compared to healthy cells. Whilst these cells maintained exclusion of Trypan Blue there was a correlation between the numbers of these non-circular cells and a reduction in viability (Figure 4.13). Viability was recorded using the total of dead cells determined by the Trypan Blue exclusion assay and using the number of non-circular cells and added to that figure to ensure accurate viability counts. Analysis of data using both counting parameters arrived at the same results. Puromycin selection pressure was set at 12.5μg/mL in order to kill non-transfected cells within 72-96 h as opposed to 10μg/mL that would take up to 120 h.

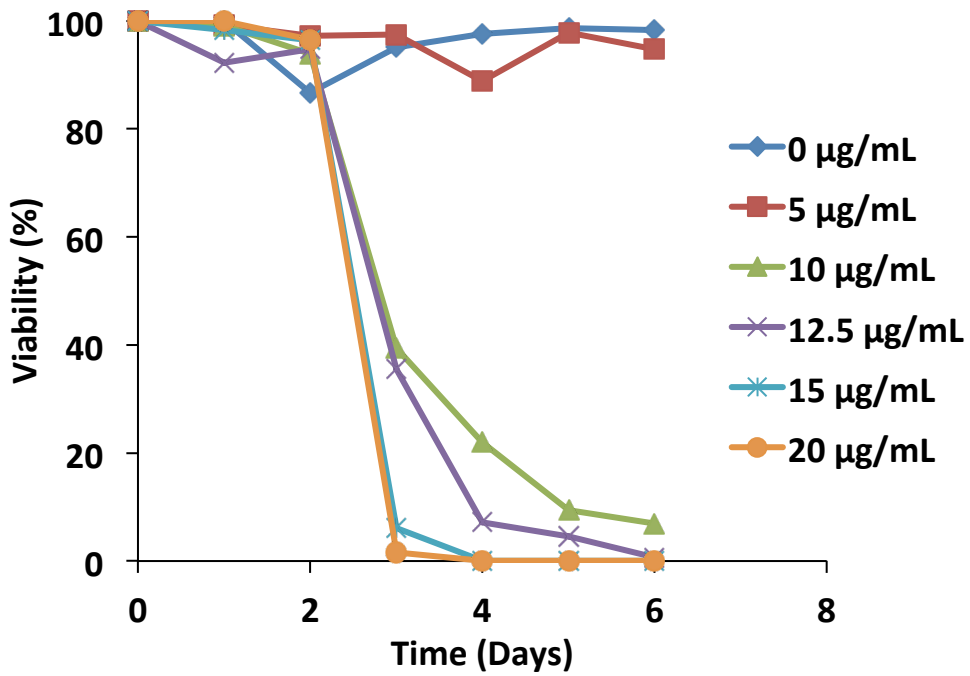


Figure 4.12 Viability of CHO-S cells under puromycin exposure – cells excluding Trypan Blue. Selection of stably transfected CHO cells required the use of puromycin antibiotic; hence it was necessary to titrate the appropriate concentration to kill non-stable cells. Suspension adapted CHO-S cells were cultured to $1x10^6$ cells/mL and 100% viability in 30mL of CD-CHO media. Cultures were exposed to puromycin at concentrations of 0, 5, 10, 12.5, 15 and 20 µg/mL and the viability of cultures determined by the Trypan Blue exclusion assay. Data shows a single data set. Cells excluding Trypan Blue were counted as viable.

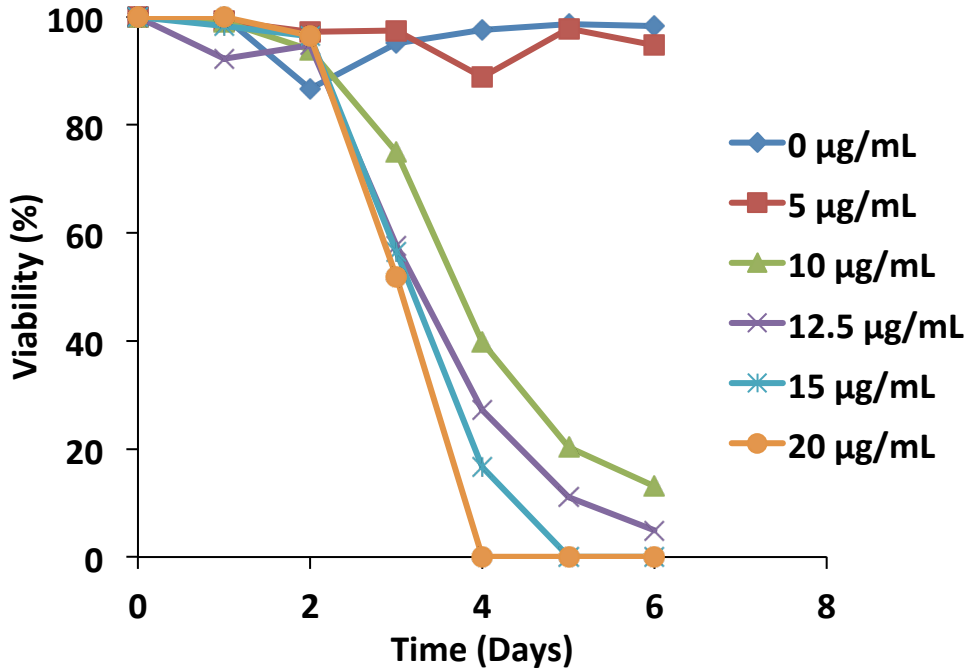


Figure 4.13 Viability of CHO-S cells under puromycin exposure – cells exhibiting normal morphology. It was noted in a previous titration that cell morphology was impacted during selection procedures and that those cultures with high percentages of non-circular cells had a reduced viability in the following days. Here viability was determined by counting both Trypan blue positive cells (blue cells) and deformed cells (non-circular) as non-viable cells to compare against the Trypan blue exclusion assay alone. The results were very similar to the Trypan blue assay alone. Data shows a single data set.

4.2.9 CHO-S transfection with SM3EL-Fc-UCOE and stable cell selection

CHO-S cells were passaged to $0.5x10^6$ cells/mL 24 h prior to transfection in 30mL of serum free Freestyle media to obtain an exponential phase culture at a density of $1x10^6$ cells/mL on the day of transfection. Transfection was carried out using linear 25 kDa PEI, using a PEI to DNA ratio of 1:2, as described in Chapter 3. Cells were counted every 24 h and the percentage viability determined via the Trypan Blue assay. As observed previously, cell growth rate reduced after transfection compared to non-transfected controls. 48 h post transfection the control population had a greater cell density than transfected cells (2.8 and $1.97x10^6$ cells/mL respectively) whilst viability was 100% compared to ~95% (Figure 4.14 A and B). The culture was centrifuged at 150x *g* and suspended in fresh media supplemented with 12.5 µg/mL puromycin. The effects of puromycin incubation were observed 24 h after

addition. The viability of in both groups dropped to ~80% even as cells continued to divide for a further 24-48 h. The viability of control and transfected cultures decreased rapidly after Day 4 and all cells were non-viable at Day 7. Puromycin selection pressure of 12.5μg/mL resulted in unsuccessful stable cell selection.

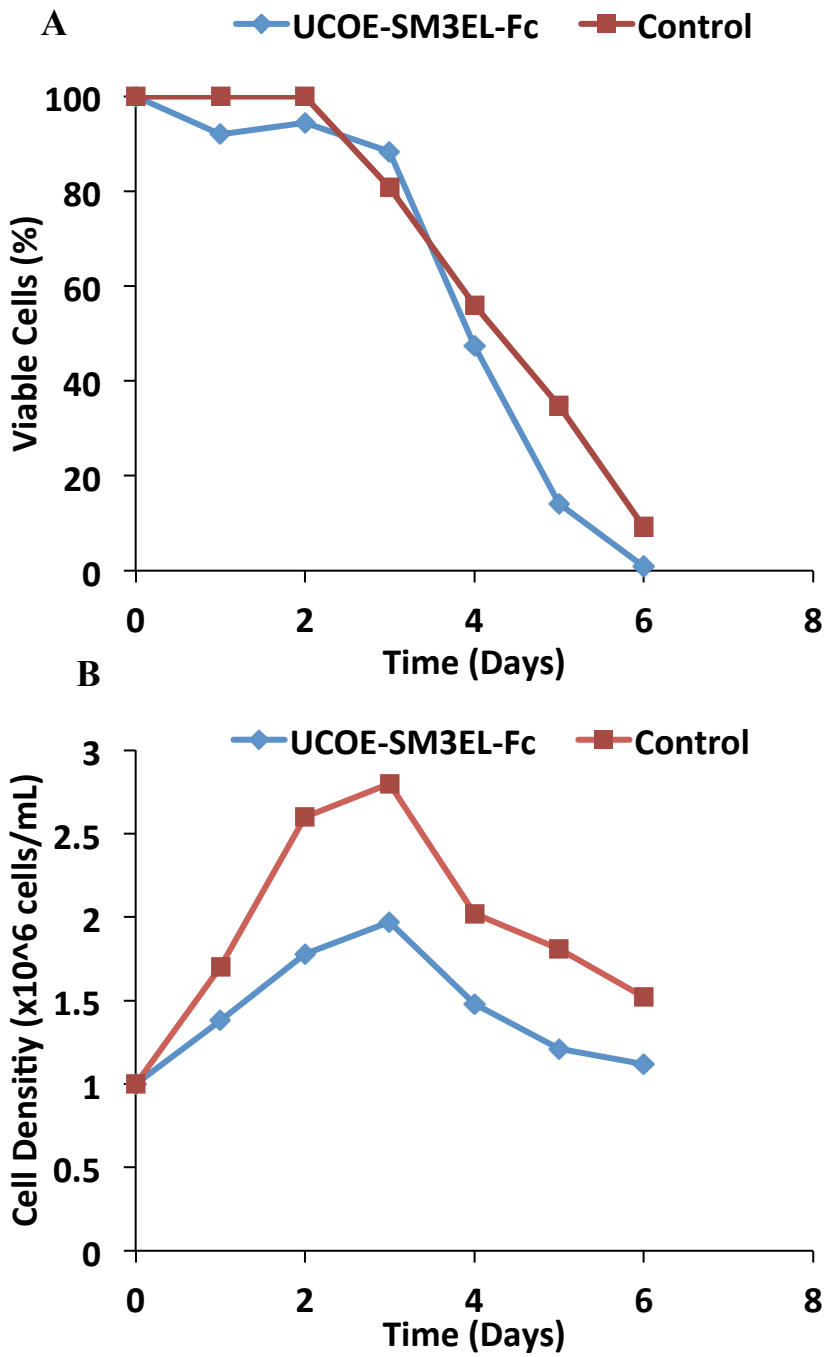


Figure 4.14 Selection of CHO cells transfected with pSM3EL-Fc-UCOE. CHO-S cells at a density of $1x10^6$ cells/mL were transfected using 25 kDa linear PEI with plasmid DNA coding of the scFv-Fc under the control for the UCOE expression vector. After 24 h puromycin selection reagent was added to a final concentration of 12.5 µg/mL to both the transfected cells and a non-transfected control culture. After 6 days post-transfection both control and transfected cells were dead despite maintaining growth up to day 3. A) Cell viability was determined using the Trypan Blue exclusion assay. B) Cell density was determined by counting cells plated in a haemocytometer.

4.2.10 Stable cell selection using a graduated puromycin selection ramp

A gradual increase of the selection concentration was employed after communications with the manufacturer. Using a graduated increase in selection pressure was hypothesized to allow cells to adapt to puromycin conditions without immediately being exposed to the upper limit of puromycin tolerance before sufficient expression of the resistance gene or recovery after transfection. Cells were cultured and transfected as described previously. After a recovery period of 24 h puromycin was added at 5 μg/mL, with subsequent addition of 2.5 μg/mL every 24 h until a final concentration of 12.5 μg/mL. Control cells maintained greater viability and cell division rates compared to transfected cells during the recovery phase, however upon graduated puromycin addition cell division slowed. There was a slower decrease in viability compared to higher puromycin concentrations (Figure 4.14 and 4.15). Nonetheless, both cultures rapidly decreased in viability after concentration reached 24 h exposure to 12.5 μg/mL puromycin. At Day 9 there was no difference between transfected and control cell populations and the selection was terminated.

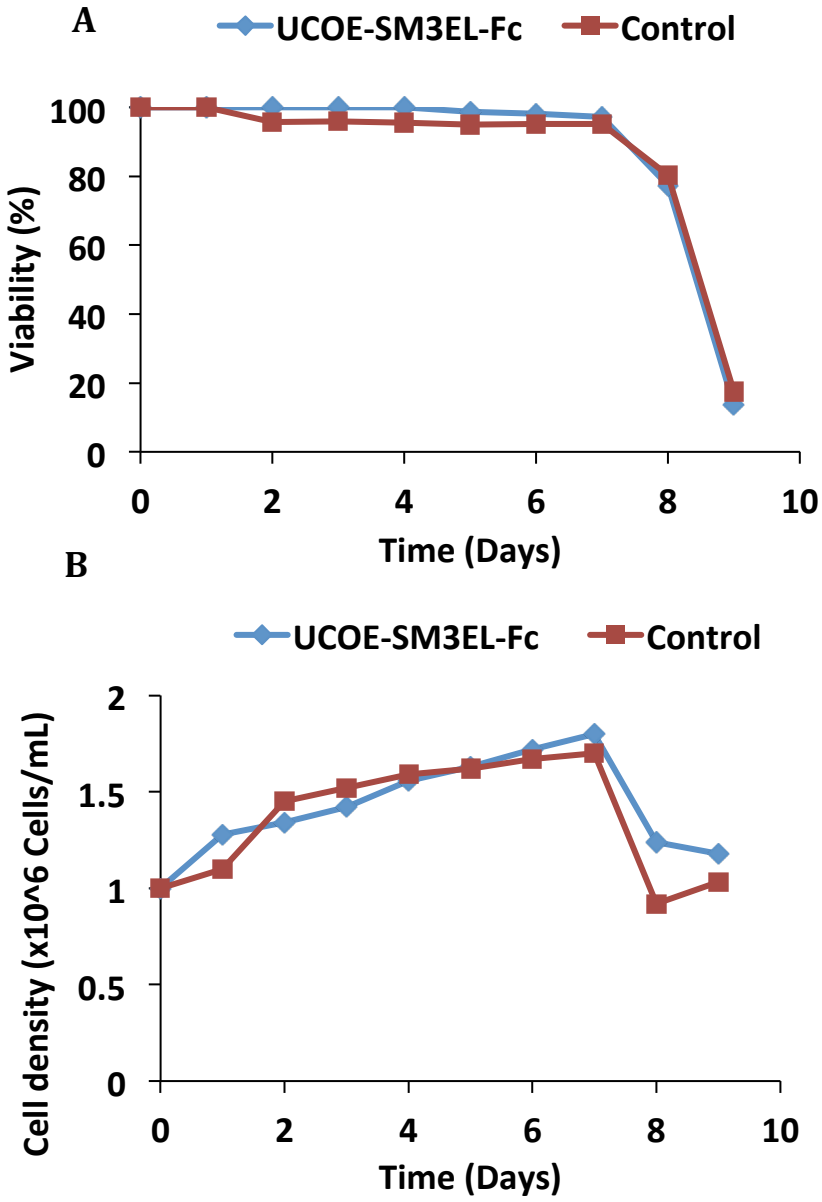


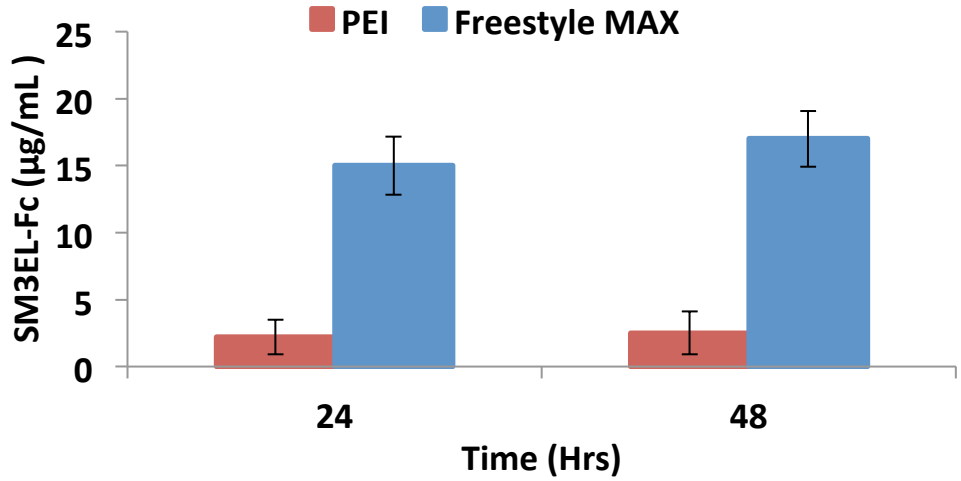
Figure 4.15 CHO cells under graduated puromycin selection after transfection with pSM3EL-Fc-UCOE. CHO-S cells at a density of $1x10^6$ cells/mL were transfected using 25 kDa linear PEI with plasmid DNA coding for the scFv-Fc. After 48 h puromycin selection reagent was added to a final concentration of 5 µg/mL to both the transfected cells and a non-transfected control culture. The selection concentration was increased every 24 h by 2.5µg/mL until reaching a final concentration of 12.5µg/mL on day 5. Cell cultures were suspended in fresh selection 12.5 µg/mL selection media on day 7 after which cell viability in both control and transfected cultures rapidly declined. A) Cell viability was determined using the Trypan Blue exclusion assay. B) Cell density was determined by counting cells plated in a haemocytometer.

4.2.11 Analyses of SM3EL-Fc expression by CHO cells after transfection using PEI or Freestyle MAX transfection reagent

After unsuccessful selection attempts using the PEI and CHO-S cells the amount of scFv-Fc produced by cultures was analysed by ELISA. These results showed that a small amount of scFv-Fc was detectable after 24 and 48 h (both <3μg/mL) from cultures transfected with PEI. However the minimal amount of protein expression suggested that low transfection efficiencies might have affected the number of potential integration events and as such potential for stable selection. A comparison between transfection efficiency of PEI and the more expensive Freestyle MAX reagent from Invitrogen was carried out as well as an assessment of protein expression.

Cell cultures were co-transfected with separate plasmids coding for the scFv-Fc and a Green Fluorescent Protein using either PEI as per the previous protocol or with Freestyle MAX according to the manufacturer's instructions. Initial (transient) expression of SM3EL-Fc was greater in cells transfected via Freestyle max reagent after 24 and 48 h (Figure 4.16A) showing substantially improved expression in the Freestyle MAX condition over PEI (16μg/mL vs. ~ 2.4μg/mL respectively). Further to this there was an increase in GFP positive cells of over 40% in the Freestyle transfected condition as determined by flow cytometry (Figure 4.16B). As a result Freestyle MAX was used for transfection of CHO cells for stable selection.

A



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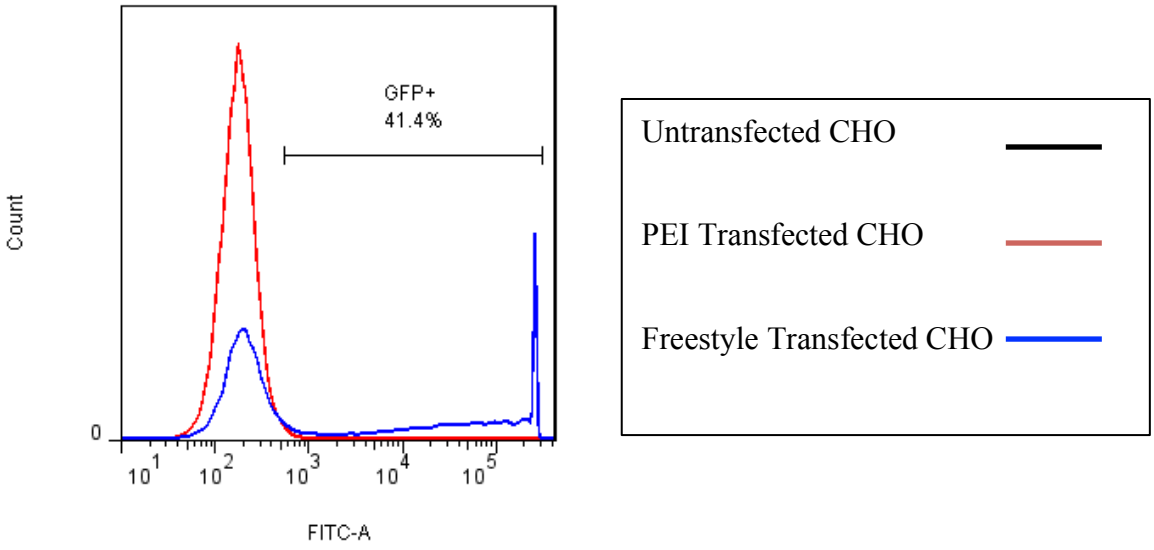

Figure 4.16 Expression of SM3EL-Fc and GFP by CHO-S cells after transfection with either PEI or Freestyle MAX. CHO-S cells at a density of $1x10^{6-}$ cells/mL were co-transfected with the scFv-Fc expression plasmid and a GFP expression plasmid using either PEI (as described previously) or Freestyle MAX (Life Technologies) (as per the manufactures instructions). A) Expression of the scFv-Fc was analysed by PTA-ELISA at 24 and 48 h post transfection. Cultures transfected with the Freestyle MAX reagent produced substantially more scFv-Fc than those transfected with PEI (16 µg/mL and ~2.4 µg/mL respectively). Data shows mean scFv-Fc in supernatant and error bars represents one standard deviation away from the mean (n=3). B) Analysis of Green Fluorescent Protein expression in transfected cells as determined by flow cytometry shows that there was an increase of over 40% in GFP positive cells in the Freestyle transfected condition.

4.2.12 Stable cell selection after transfection with Freestyle MAX

CHO cells were transfected according to the manufacturer's instructions and a graduated selection pressure was applied as previously described (Section 1.2.4.3) after a 48 h recovery period. Control cells divided at a substantially greater rate and had improved viability compared to transfected cells immediately after transfection (Figure 4.17). However, after 10 days under selection pressure control cells were non-viable and the viability of transfected cells was ~15% in transfected cultures indicating a stable cell population had potentially been selected.

Transfected cells rapidly recovered despite being maintained under selection pressure and after Day 21 were 100% viable and dividing at a sufficient rate for cryopreservation. Cells were cryogenically frozen at low passage number (P3/4).

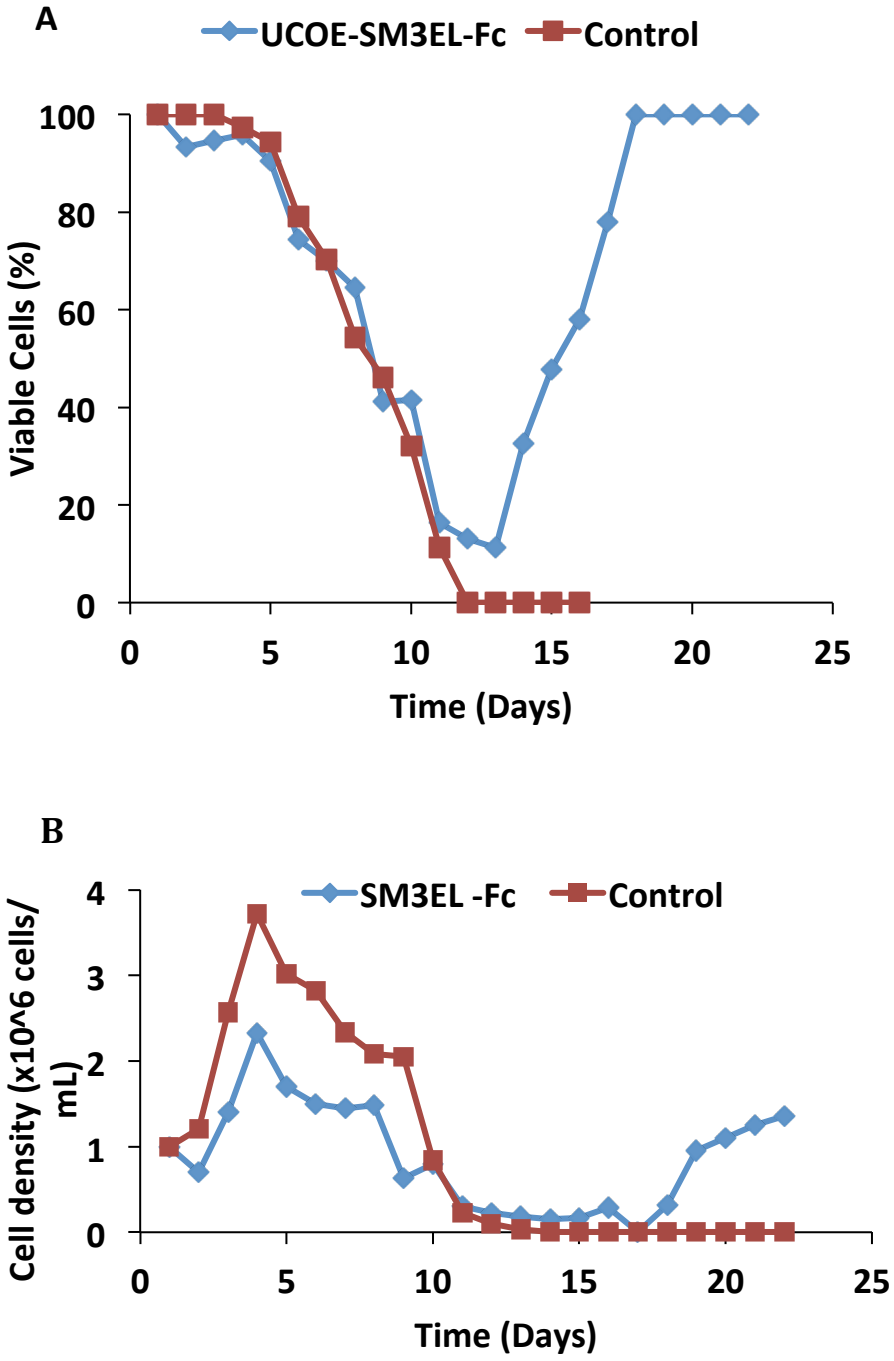

Figure 4.17 CHO cells under graduated puromycin selection after transfection with Freestyle MAX reagent. CHO cells were transfected with plasmid coding for the scFv-Fc as described previously using the Freestyle MAX reagent. Puromycin selection was increased gradually from 5µg/mL after 48 h increasing by 2.5µg/mL every 24 h until reaching a final concentration of 12.5 µg/mL. Transfected cells dropped in viability initially after transfection showing similar results to previous data with improved transfection efficiency. After 13 days under puromycin selection the non-transfected control cells were all dead whilst the transfected culture remained >10% viable. These cells rapidly recovered to 100% viability 5 days after the control cells reached 0% viable cells.

4.2.13 Selecting media for shake flask based, batch mode expression

A number of serum free cell growth media are available for batch based protein expression by CHO cells. In this study media of 3 different compositions were compared for cell growth, viability and expression of SM3EL-Fc. The stable cell line was thawed out and allowed to grow to between 1-2x10^6 cells/mL in CD-CHO media from Invitrogen (the media used during cryopreservation) before three passages in ether CD-CHO (Invitrogen), CD-CHO3 (Ex cell) or a 50:50 mix of the two (Mix 6).

Cells were grown in 30mL of each of the selected media in shake flask cultures from a starting density of 0.3x10^6 cells/mL. Both growth rate and average protein expression were analysed every 24 h. Cells grown in CD-CHO reached the highest cell density (6.57x10^6 cells/mL) despite a longer lag period, whilst cells grown in Mix 6 reached the lowest maximum cell density (5.5x10^6 cells/mL) Figure 4.18. There was no significant difference (P=0.207) in the growth of cells between the media conditions. Despite this, expression of SM3EL-Fc, as analysed by ELISA, showed that cells grown in the Mix 6 condition were the highest expressers (~24μg/mL) on day 7. The Mix 6 media was therefore selected for further production processes in shake flasks.

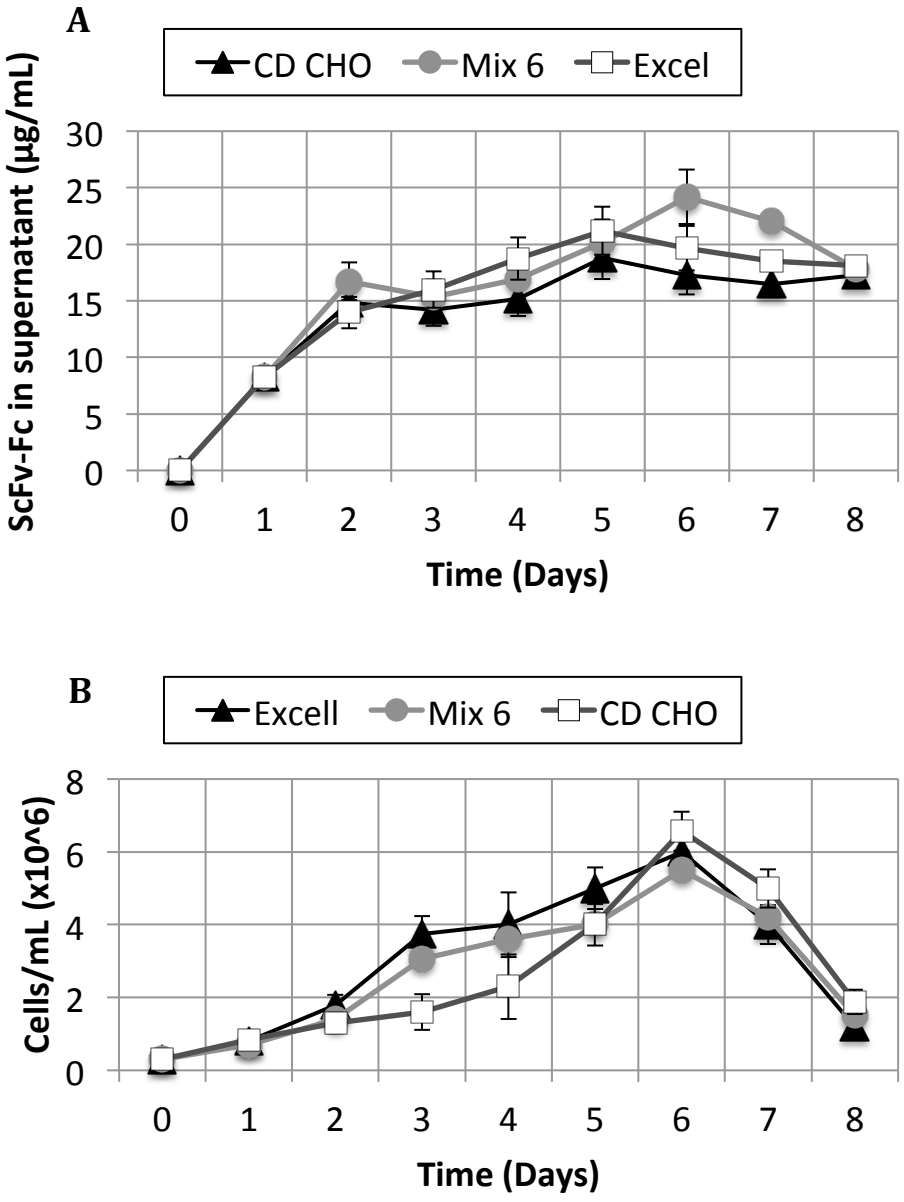

Figure 4.18 Shake flask based growth of CHO-S cells expressing SM3EL-Fc in media of three different compositions. Stably selected CHO-S cells expressing SM3EL-Fc were seeded at $0.3x10^6$ cells/mL in different media compositions and the growth and productivity analysed over time. Three media compositions were compared consisting of CD-CHO (Life Technologies), ExCell CD-CHO (Sigma Aldrich) or a 1:1 mix of both media (Mix6). There was no significant difference between the growth of cells (P= 0.207) but cells grown in Mix6 yielded the highest overall scFv-Fc expression. A) Cell density ($x10^6$ cells/mL). B) scFv-Fc secreted in supernatant as determined by PTA-ELISA. Data represents the mean and error bars show one standard deviation away from it (n=3).

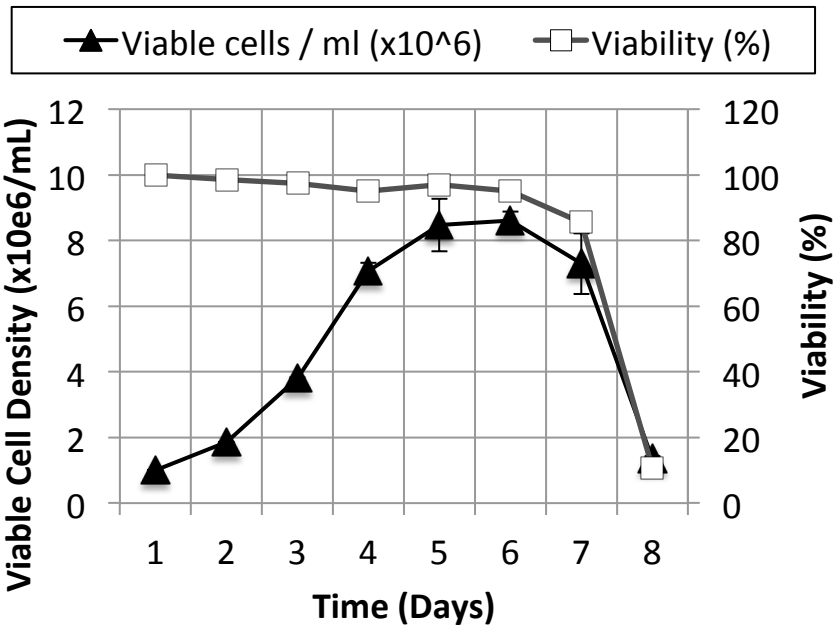

Figure 4.19 Shake flask-based batch-culture of CHO-S cells expressing SM3EL-Fc. CHO-S cells selected for stable expression of SM3EL-Fc were seeded at $0.5x10^6$ cells/mL in Mix6 media. Cells were cultured until viability dropped below 80% (day 8). The growth and viability of cells was analysed over time using the Trypan Blue exclusion assay. The cultures reached an average of $8.6x10^6$ cells/mL on day 6 and viability remained at or above 90% until day 7 after which the viability dropped dramatically to ~10%.

Shake flask culture of CHO cells stably expressing SM3EL-Fc

Shake flask culture of SM3EL-Fc expressing cells was carried out in 300mL flasks to compare maximal expression of the protein between fed-batch and batch-based expression. Cells were seeded in Mix 6 media at a density of $0.5x10^6$ cells/mL and after Day 3 were either given EfficientFeed B (3% of total volume per 24 h) or no addition (control).

Both cultures entered log phase on Day 3 reaching ~$3.7x10^6$ cells/mL at 100% viability. Batch cultured cells reached a maximum of $8.6x10^6$ cells/mL (Figure 4.19) before viability dropped rapidly after day 6. Cells supplemented with feed remained over 90% viable for a further 4 days reaching a maximum viable cell density of $1.28x10^7$ cells/mL (Figure 4.20). Specific growth rate was higher during log phase in fed batch cultures compared to batch cultures (0.56 to 0.5/day respectively) whilst specific productivity was also higher in the fed batch culture (2.96 compared to 4.41

pg/cell/day). Supplemented cultures not only reached a higher cell density but also produced more scFv-Fc compared to the control producing a maximum of 86.6μg/mL (Figure 4.21) compared to 34.3 μg/mL in batch based culture.

Protein from fed-batch cultures was purified via protein A MabSelect chromatography after centrifugation and filtration before concentration and dialysis into PBS overnight (see Methods) for further analysis.

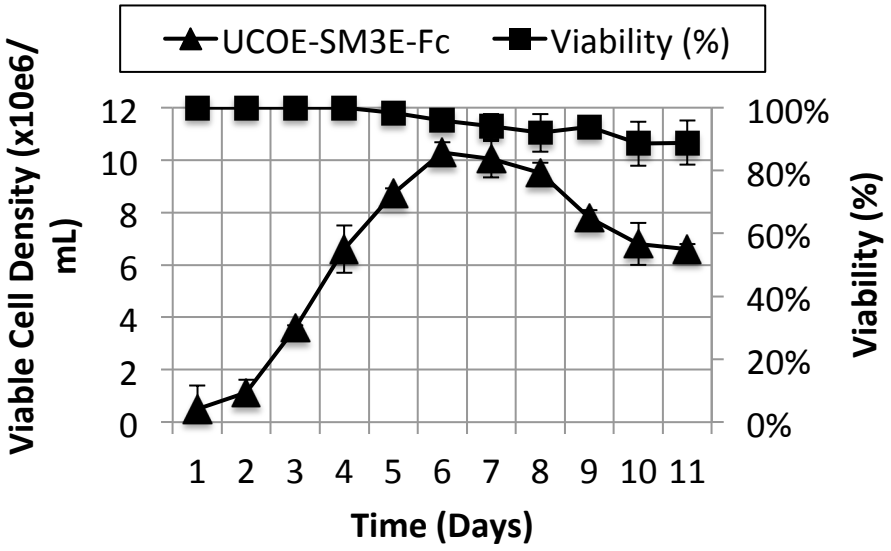

Figure 4.20 Fed-Batch culture of CHO-S cells expressing SM3EL-Fc. CHO-S cells stably expressing SM3EL-Fc were grown in 300mL Mix6 media in shake-flasks. On day 4 cultures were supplemented with 3% final volume of CD-CHO EfficientFeed B (Invitrogen) or a necessary to obtain media with greater than 4g/L glucose. Cell density and viability were determined using the Trypan Blue exclusion assay. Cells reached a maximum density of 10.28×10^6 cells/mL and remained above 88% viability throughout the culture period.

Protein from stable CHO and HEK293F transient expression was compared to assess difference in protein stability and *in vitro* efficacy. The stability of proteins from transient and stable expression in the two host systems was analysed using SDS-PAGE, repeated freeze-thaw analysis and differential scanning fluorimetry as well as an *in vitro* complement-dependent cytotoxicity assay to assess effector function.

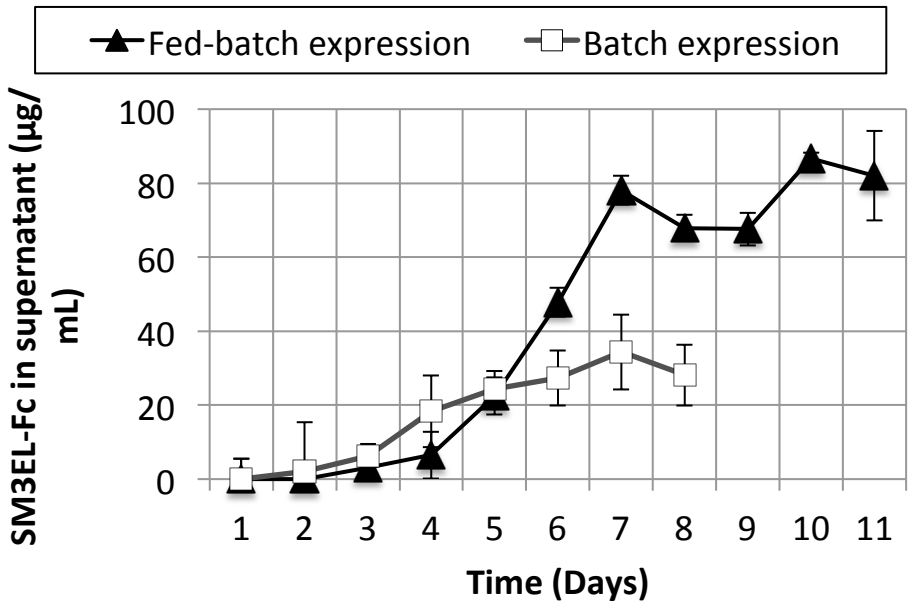

Figure 4.21 Expression of SM3EL-Fc by batch and fed-batch cultured CHO cells. CHO-S cells stably expressing SM3EL-Fc were cultured in 300mL of Mix6 media in shake flasks. After day 4 fed-batch flasks were given 3% final volume supplement of CD-CHO EfficientFeed B whilst batch culture flasks were not supplemented. Daily samples were removed and solids separated before storage of the supernatant at -80°C for analysis of scFv-Fc content by ELISA. Batch based culture reached a maximum of 34.4µg/mL compared to maximal expression of 86.6µg/mL in the fed-batch condition.

4.2.14 SDS-PAGE freeze thaw analysis

ScFv-Fc was diluted to 1mg/mL in PBS buffer prior to freeze-thaw analysis. Protein was frozen to -80°C and thawed out at room temperature over 24 h cycles. Samples were removed and boiled in reducing buffer before storage at -20°C. Protein resolution using a 10% SDS-PAGE gel ( ) showed that there was no difference between the samples of scFv-Fc from transient or stable cells. Furthermore there was also no difference between the sizes of bands resulting from repeated freeze-thaw cycles in either transiently expressed or stably expressed samples.

4.2.15 Stability comparison of scFv-Fc from CHO and HEK293f cells

DSF was utilised to assess and compare the Tm_{50} of proteins from the various expression systems (Figure 4.23). There was no difference found between the Tm_{50} of proteins assessed, including the peak heights and Tm50°C. This result further corroborated previous findings suggesting that the characteristics of protein from transient systems was a good indicator of final performance of the product after stable cell expression.

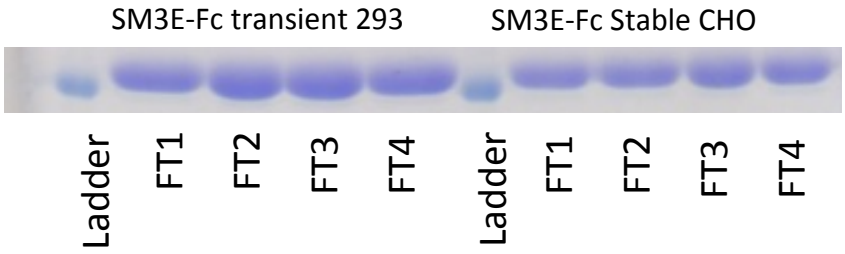

Figure 4.22 SDS-PAGE separation of scFv-Fc from transient HEK293F and stable CHO-S expression. scFv-Fc was transiently expressed by HEK293F cells and stable CHO-S cells in shake flask conditions. Protein was purified using protein A MabSelect chromatography (GE Healthcare) and dialysed into PBS and diluted to 1 mg/mL. Samples were frozen at -80°c and thawed out at room temperature in cycles of 24 h. After each cycle samples were removed and heated to 100°c for 5 min in reducing buffer before storage at -20°C. There was no difference in the size of resolved bands of scFv-Fc from either condition using 10% SDS-PAGE resolution, and no breakdown observed after consecutive freeze-thaw cycles. FT refers to freeze-thaw cycle number. Ladder shows 50 kDa band of a SeeBlue Ladder from ThermoFisher (UK).

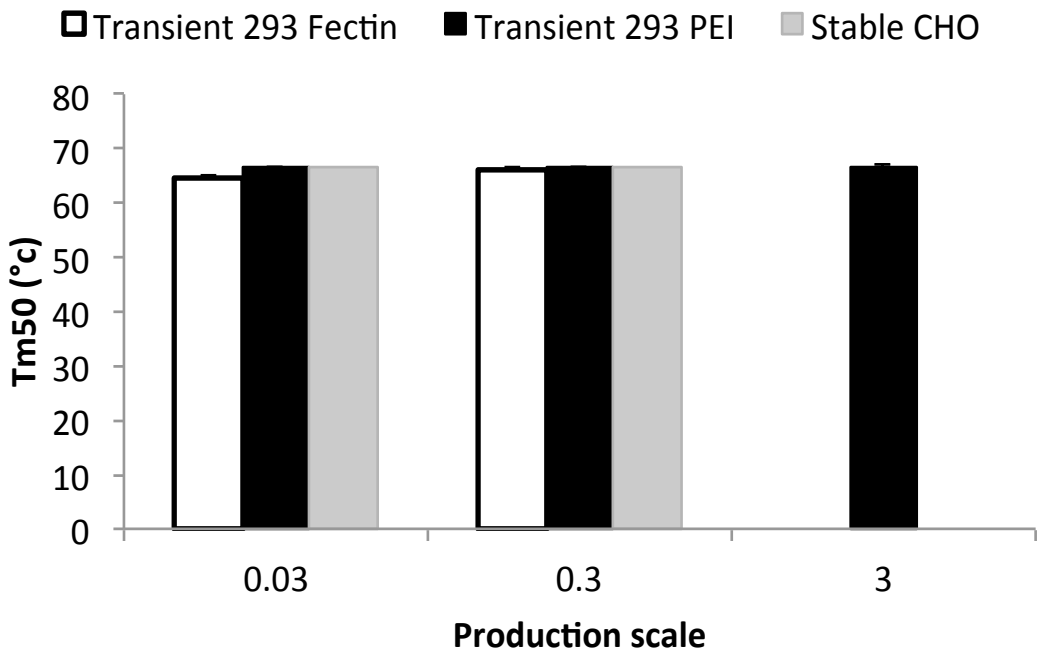

Figure 4.23 Thermal stability of SM3EL-Fc produced by transient expression in HEK293F cells and stable expression in CHO cells. Purified scFv-Fc from each scale was diluted to 1mg/mL and SYPRO orange added as described previously. Temperature-dependent protein unfolding was assessed via differential scanning fluorimetry. Comparing the Tm50 (°C) showed that there was no difference in protein stability from the different expression systems. Data shows mean and error bars of one standard deviation (n=3).

4.2.16 Comparison of functional *in vitro* activity of SM3EL-Fc from CHO stable and transient HEK293F cells

Comparative analysis of the structural integrity and thermal stability of scFv-Fc showed that proteins from both sources of expression behaved in a very similar way with no differences in characterisation assays. However these assays do not necessarily translate into good efficacy in a therapeutic setting. In order to further assess the effector function of the constructs the complement dependent cytotoxicity (CDC) activity was compared using the MTS Tetrazolium assay utilising cells both positive and negative for the target and rabbit complement.

Capan-1 cells (expressing CEA) and Hela cells (negative control) were plated out in 6-well plates ($1x10^5$ cells). 24 h after seeding, SM3EL-Fc was added to the cells at a final concentration of 1μg/mL and the plate was incubated at 4°C to stop internalisation. After 1 h, baby rabbit complement was added at a concentration of 1μg/mL and the complement mediated cell killing allowed to take place under normal incubation conditions for 1 h. Cell viability was analysed via the reduction of MTS Tetrazolium to form a yellow coloured substrate in the media. This was analysed using a 6 well plate reader via absorbance at (490) nm.

The results showed that when either transient or stably expressed SM3EL-Fc and complement were added to cells the viability of CEA expressing capan-1 cells was reduced by over 50%. There was no reduction in the viability of Hela cells suggesting that SM3EL-Fc did not have off target affects and that it successfully mediated CDC activity. When either transient or stably expressed SM3EL-Fc and not complement was added to the cells there was a slight reduction in viability compared to control cells with no addition. However, this effect is enhanced when complement only is added (Figure 4.24).

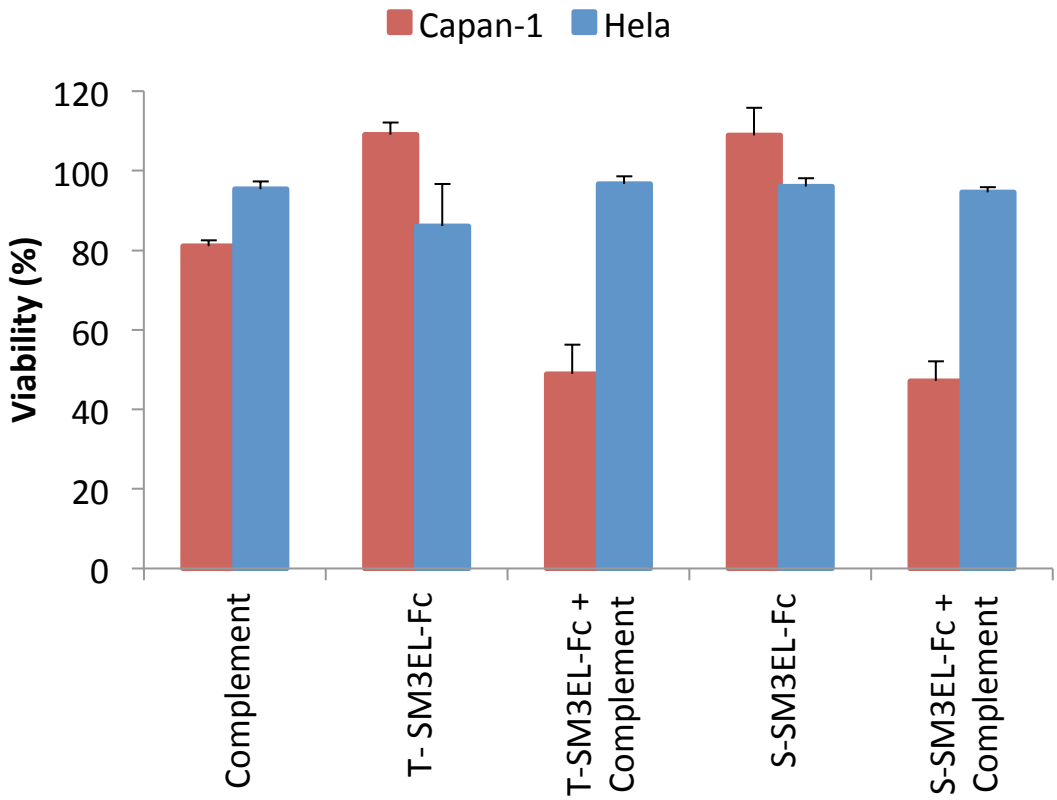

Figure 4.24 *In vitro* complement mediated cytotoxicity (CDC) activity of transient and stably expressed SM3EL-Fc. CEA-expressing capan-1 cells and CEA-negative Hela cells were plated out in a 96-well tissue culture plate (10,000 cells/well). After 24 h 1µg of SM3EL-Fc was added to each well and the plate was incubated at 4°C for 1 h. Rabbit complement (AbD Sertech) was added (11µL/well) and the plate incubated for a further 1 h at 37°C followed by MTS (Cell Titre 96 Aqueous One Solution, Promega) assay substrate (12µL/well) and an incubation for 30min at 37°C. Analysis of colour was carried out using by absorbance at 490nm in a Varioskan Flash 96well plate reader. There was a reduction in the viability of capan-1 cells of ~52% with both transiently expressed (HEK293F) or stably expressed (CHO-S) scFv-Fc, showing no difference between the two samples. There was also no reduction in the viability of Hela cells further confirming no off-target effects of the protein. Figure shows mean data and error bars are one standard deviation (n=3).

4.3 Discussion

Clonal selection of stable cells is often used to obtain high expression of a recombinant protein. However this is a laborious process that can take several months creating a bottleneck in the development process. The aim of this chapter was to achieve increased expression of the model scFv-Fc by generating a pool of high expressing stable cells that could be cultured for protein production after primary selection. Two different transgene integration techniques were investigated relying on either targeted transgene integration or manipulation of heterochromatin structure and promoter activity rather than standard random integration. Media selection and feeding strategies were also used to maximise recombinant protein expression within the lab environment.

Random integration of transgenic DNA into host cells is currently the standard method of obtaining stable mammalian cell lines. Whilst this method does generate stable integrants, it has a number of draw backs for rapid expression such as producing a pool of highly heterogeneous cells with a wide variety of transgene copy number, integration loci and gene silencing levels (Liu et al., 2005). This heterogeneity was illustrated here using random transgene integration into Hela cells resulting in sub-cloned populations with a range of protein expression, growth and recovery characteristics.

High expressing cells are usually obtained from a pool of stable integrants using antibiotic or metabolic selection procedures; however these methods are often labour intensive, costly and inefficient. Indeed the use of antibiotic selection methods may, in itself, effect transgenic protein expression (Kaufman et al., 2008). During early developmental research of recombinant therapeutics a number of candidates are investigated and characterised without the need for cGMP based clonal production. However screening of huge numbers of cells is still a primary method for isolating high expressers at this stage. As such, a system for rapid generation of a pool of high expressing cells that can be used to produce substantial quantities of recombinant proteins at pre-GMP stages is highly desirable.

A number of approaches have been developed that go someway to addressing these issues; these include synchronisation of cells prior to transfection using mimosine (Grosjean et al., 2002a), aphidicolin or butyrate (Golzio et al., 2002) in order to achieve improved transfection rates, use of weak or attenuated antibiotic promoters to ensure selection of only very high expressers (Chin et al., 2015; Hitoshi et al., 1991) and codon-usage optimisation (Inouye et al., 2015). However, whilst these methods do enhance protein expression they do not actively address the issue of transgene integration.

The use of recombinase-mediated site specific integration technology (a.k.a recombinase mediated cassette exchange) has been reported as far back as 1988 (Wirth et al., 1988). The proposed advantages of using this system are that, in cells with well-characterised integration sites, transfected populations have the transgene integrated in the same locus, with the same expression profile. Hence, the resulting stable integrants are isogenic for the transgene negating the need for further selection once stable cells are obtained. The Flp recombinase system in particular has been used with success in the development and study of isogenic hosts in a number of cell lines (Liu et al., 2005). Alternative genetic engineering techniques have also been developed that may allow this approach to mature fully in high expressing cell development including the use of Cre recombinase and CRISPR mediated genetic modifications (Lee et al., 2015).

In this chapter Hela cells were used as a model system to study flp-recombinase mediated generation of isogenic cells. Hela cells are a widely used cell line that was used here to model expression characteristics with the intention of developing the system into other suspension cell lines. Whilst Hela is a readily accessible cell line, and the modified H2Z version described here was made in house, there are a number of commercially available Flp-In cell lines with well characterised integration sites specifically for generation of high expressing cells including CHO, BHK and 293 cells (Life Technologies).

The second technique investigated in this chapter was the use of a ubiquitous chromatin opening element-based (UCOE) vector. The UCOE element utilises a composite set of promoters for ‘house keeping’ genes that not only maintain a high

level of expression but are also resistant to heterochromatin-mediated gene silencing (Antoniou et al., 2003) to generate cells with stable high production of the target protein. The combination of an extended CpG island along with a composite dual promoter consisting of those for house keeping genes and the hCMV promoter has been shown to have significant improvements of protein expression irrespective of the location of transgene integration (Williams et al., 2005). In previous work transgene expression levels in cell pools have been shown to increase significantly when compared to other expression vectors (Boscolo et al., 2011).

In this chapter the model scFv-Fc was incorporated into the UCOE system and used in conjunction with suspension adapted CHO cells. As discussed previously CHO cells are the industry standard for recombinant protein expression and, without the need for transient expression, were chose as the final host system. In order to remain a cost effective expression system PEI was initially used as the transfection reagent, however selection of cells proved unsuccessful. After analysis and comparison of transfection efficiency of PEI and Freestyle MAX (Life Technologies), the more expensive FreestyleMAX reagent was used instead resulting in both improved transfection and successful selection of stable cells.

Having established a pool of stable transfectants, maximal expression was analysed in shake flasks using batch-based culture of cells. The results from transfected cell pools in this chapter yielded an expression level of up to 34μg/mL in batch operation rising to ~86 μg/mL in fed-batch mode. Clonal selection of cells with UCOE enhanced scFv-Fc expression has previously indicated that pooled cells and monoclonal cells expression levels were comparable (Boscolo et al., 2011). Therefore in early biopharmaceutical development stages, when monoclonal expression is not a necessity, a polyclonal population would carry out the same production role without the time and costs associated with clonal selection.

Further increases in protein production can be easily achieved using extended cell culture practises such as fed-batch or perfusion-based techniques. However whilst perfusion can provide exceptionally high yields of protein (Lee et al., 2012), it also requires significant outlay in terms of specialist equipment and limited scalability. In contrast, fed-batch based culture requires only the addition of an enriched feed media

making it an accessible technique for many laboratories. The results in this chapter showed that that the amount of recombinant protein in fed batch-based culture was almost 40% greater than that of batch-based culture, even without process optimisation. Further to this, the stable pool yielded an increase of over 12% compared to transient based expression.

The use of TGE to express the first quantities of recombinant proteins prior to committing to stable expression relies on the product accurately represent the characteristics of protein from the intended final expression system. Transient and stable expression of recombinant proteins, especially antibody based fragments, has been investigated previously showing that there is good correlation between the stability, effector function and expression profile of protein from both systems (Diepenbruck et al., 2012). However the relationship between the characteristics of the same protein expressed in different systems has not been fully investigated, save for glycosylation differences and yield.

Here, scFv-Fc from the transient and stable expression systems described in chapters 3 and 4 were compared in terms of stability and effector function. The respective cell lines are of Human or Chinese Hamster origins; however both are routinely used at for protein expression purposes. Protein stability as determined by differential scanning fluorimetry and repeated freeze-thaw cycle SDS-PAGE showed that there was no difference between proteins expressed by either system at scales up to 300mL. Assessment of effector function, a key determinant of protein performance, indicated that the difference in CDC activity of the proteins was not significant ($p > 0.1$). The results presented here suggest that, whilst there may be differences in terms of glycosylation structure (Croset et al., 2012), the stability and effector function of the model protein was unchanged between the two systems. Hence, there is potential for early recombinant protein work to be carried out rapidly in a transient expression system with the results remaining relevant to a different final expression system allowing a more rapid and flexible approach to early protein expression.

Conclusion

The aims of this chapter were to develop a system that would rapidly establish high expressing stable cells for use in early protein production and to compare products from transient and stable expression. This was achieved using a combination of a UCOE expression vector, fed-batch feeding and selection of an efficient transfection reagent to enable commencement of production within 2 weeks.

A comparison of the scFv-Fc produced in both transient and stable cell based systems established that there was no difference in the stability or effector function of the respective proteins. These results show that the characteristics of a transiently expressed scFv-Fc from HEK293 cells can be generalised to the same protein expressed in stable CHO cells reducing the necessity for stable cell protein screening.

Chapter 5 Primary recovery of recombinant proteins from unclarified mammalian cell feed streams using radial flow chromatography and large bead IMAC resin*.

5.1 Introduction

Current methods for the capture of recombinant proteins require the separation of solids from liquids to remove cells and debris prior to capture based on ion exchange or affinity. The work in this chapter describes a new method developed to address these issues. The method exploits large bead diameter chromatography resins utilising a stationary phase made of agarose beads typically between 300-500μm in diameter packed into a radial flow column.

Solid-liquid separation is the crucial first step in most mammalian cell bioprocesses and has been impacted significantly by recent improvements in upstream technological advances. Primary processing of high cell density feed streams requires particular consideration to avoid such cell stresses and shearing that could result in protein aggregation and cell lysis (Hutchinson *et al*., 2006; Kiese *et al*., 2008). The latter affects the quantity and quality of product through contamination with host cell protein (HCP), protease and DNA (Sandberg *et al*., 2006).

The capture method proposed here is hypothesized to facilitate recovery from unclarified feed, thus negating traditional solid-liquid separation stages prior to chromatographic capture. Large diameter resins create a semi-porous packed bed enabling simultaneous protein capture and concentration whilst allowing cells to pass through. As with many resins, a generic scaffold such as agarose can be functionalized in a number of ways meaning that affinity capture, IMAC or ion exchange chromatography can be used making this processing technique widely applicable to many recombinant proteins.

The traditional axial flow column, where feed and pressure are both perpendicular to the packed bed, is used in most recovery processes. However bed height at scale can affect performance meaning that significant footfalls and high-pressure drops are necessary. An alternative to axial flow is radial flow chromatography. In contrast to

axial flow, RFC consists of two concentric cylindrical porous frits holding a stationary phase between them. In RFC, feed flows from the outer to the inner surface across the radius of the column (Figure 5.1) providing a minimal pressure drop. Improved elution peak resolution due to the trapezoidal column geometry and greater operational flow rates are reported as advantages (Besselink *et al*., 2013). In this chapter large diameter immobilized metal affinity chromatography (IMAC) beads were packed into a scale down RFC system.

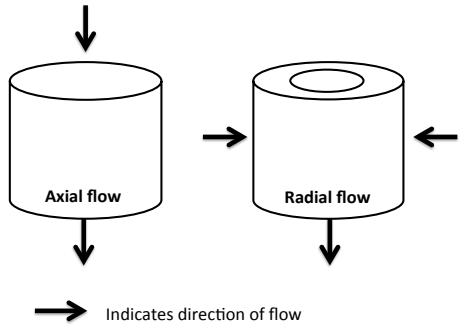

Figure 5.1 Schematic of axial and radial flow chromatography systems. Axial and radial flow columns with respective directions of feed flow from top to bottom or outer surface to inner surface.

Unlike proteins with Fc fragments, which have a natural affinity for Protein A from *Staphlycocuss aureus*, most recombinant proteins require addition of a molecular tag to facilitate affinity capture. Many tags are available to facilitate capture however the polyhistidine repeat tag remains the major workhorse for tag-based purification due to the simplicity of amino or carboxy terminal fusion and purification of proteins that lack a natural purification tag (Terpe, 2003).

In this chapter a His-tagged glycoprotein, the CEA, was expressed in CHO cells using the UCOE expression system discussed in Chapter 4. CEA is a clinically relevant glycoprotein used in many diagnostic and therapeutic applications (Chester et al., 2000; Francis et al., 2002; Graff et al., 2004; Li et al., 2010, Miranda-Rota., in preparation) as well as the molecular target of SM3EL-Fc as expressed in previous chapters.

Here, a process for protein recovery from unclarified mammalian cell fermentation streams was developed using a RFC packed with a large diameter resin to obtain an integrated primary capture step.

The method was developed using suspension-adapted CHO cells grown in shake flask culture. The process was then scaled up to a 3L bioreactor whilst maintaining chromatography bed height. The study forms the basis of a primary recovery step applicable to the recovery of many recombinant proteins from unclarified mammalian cell feed streams.

Aims

Develop a process for protein recovery directly from unclarified mammalian cell feed streams and demonstrate reactor harvest integration

Chapter objectives

- Show that CHO cells can survive passage through a scale down radial flow column packed with large diameter beads.
- Optimise conditions for IMAC protein capture from model and cell containing feed streams.
- Compare recovery of HIS-tagged CEA from unclarified and clarified feeds.
- Integrate primary recovery into reactor harvesting and assess robustness.

5.2 Results

5.2.1 Design of Scale Down Radial Flow Column

Colum design was influenced by a number of factors such as frit porosity and scale down ratio (Fig 2.1). The average diameter of CHO cells (14.02μm -15.21μm (Han *et al*., 2006)) and the diameter of the beads were both limiting factors on frit pore size. Pores over 300μm would allow the resin to escape whilst pores too small would not let individual or aggregated cells pass through the column causing fouling. CHO cell aggregates in shake flask cultures did not exceed 30μm in previous shake flask cultures hence pores of 40μm and 200μm were chosen for pilot experiments using shake flask feed stocks of CHO-S at a density of $1x10^6$ cells/mL (97.4% viable). Whilst both these pore sizes would theoretically allow for cell passage it was hypothesized that the 40μm pores would create areas of high shear and thus cell disruption. Results from these experiments showed that feed passed through the 40μm pore size frit dropped in terms of cell density and had a reduced (19.38% +/-2.3%) viability indicating cell shearing during column passage as determined by the Trypan blue exclusion assay. The average size of particles in the column flow through and the total count was also substantially reduced in comparison to the original feedstock (Figure 5.2). In contrast, cells remained viable (97% +/-2.2%) when the feed was passed through a 200μm porous frit accompanied by an increase in the average size of particles in the flow through. In light of these pilot results all further experiments were carried out using the 200μm porous frit.

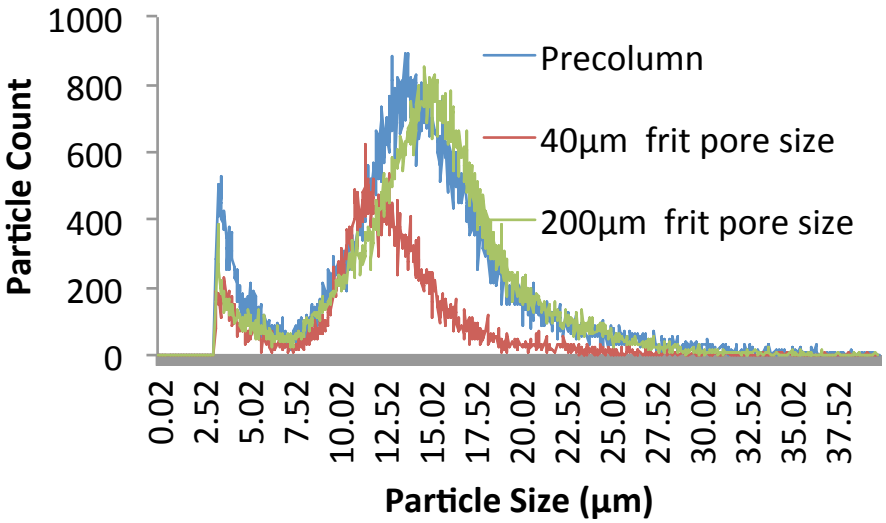

Figure 5.2 Column flow through particle size distribution of CHO cells passed through a scale down radial flow column. CHO-S cells were cultured in 300mL of CD-CHO media in shake flasks to generate a model feed stream of cell density $1x10^6$ cells/mL and viability >95%. The CHO cell feed stream was applied to the column, whilst maintaining agitation of the shake flask, at a flow rate of 1CV/min through a scale down radial flow 5mL column packed with CellThru large diameter bead resin and fitted with frits of either 40µm or 200µm pore size. The particle size distribution was analysed both prior to application of the feed stream and in column flow through samples using a CASY Cell Counter Analyser System (Roche) with appropriate dilutions.

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5.2.2 Packing of scale down radial flow column and resin charging

Column packing was carried out according to the manufacture's instructions. Packing of a radial flow bed requires careful monitoring of conditions to ensure the resin is not over pressured. Due to the trapezoidal geometry pressure characteristics are different to packing an axial column with a very rapid increase in pressure after a shorter period of low pressure as the column is progressively loaded with resin. In this chapter a micro-RFC column with 6cm bed height was loaded with Sterogene CellThru Bigbead IMAC resin.

5.2.3 CHO cell passage through radial flow column

Having established that the 200μm frits allowed an acceptable cell survival rate (97% +/- 2.2%) using a feedstock that contained a maximum of $1x10^6$ cells/mL, the effect of increased cell density was assessed. Feed streams generated in shake flask cultures were diluted to a range of cell densities from 0.5 – $8.5x10^6$ cells/mL at 100% viability reflecting the highest cell densities achieved with the stable CEA expressing cell line during previous work. Greater than 98% of cells remained viable in flow from feed streams with >$1x10^6$ cells/mL (Figure 5.3 A). This data was confirmed via analysis of cell lysis indicators including host cell DNA (hDNA) and proteolytic activity using qPCR and FRET protease assay respectively. These markers showed no significant difference between pre/post column samples ($P>0.05$) (Figure 5.3).

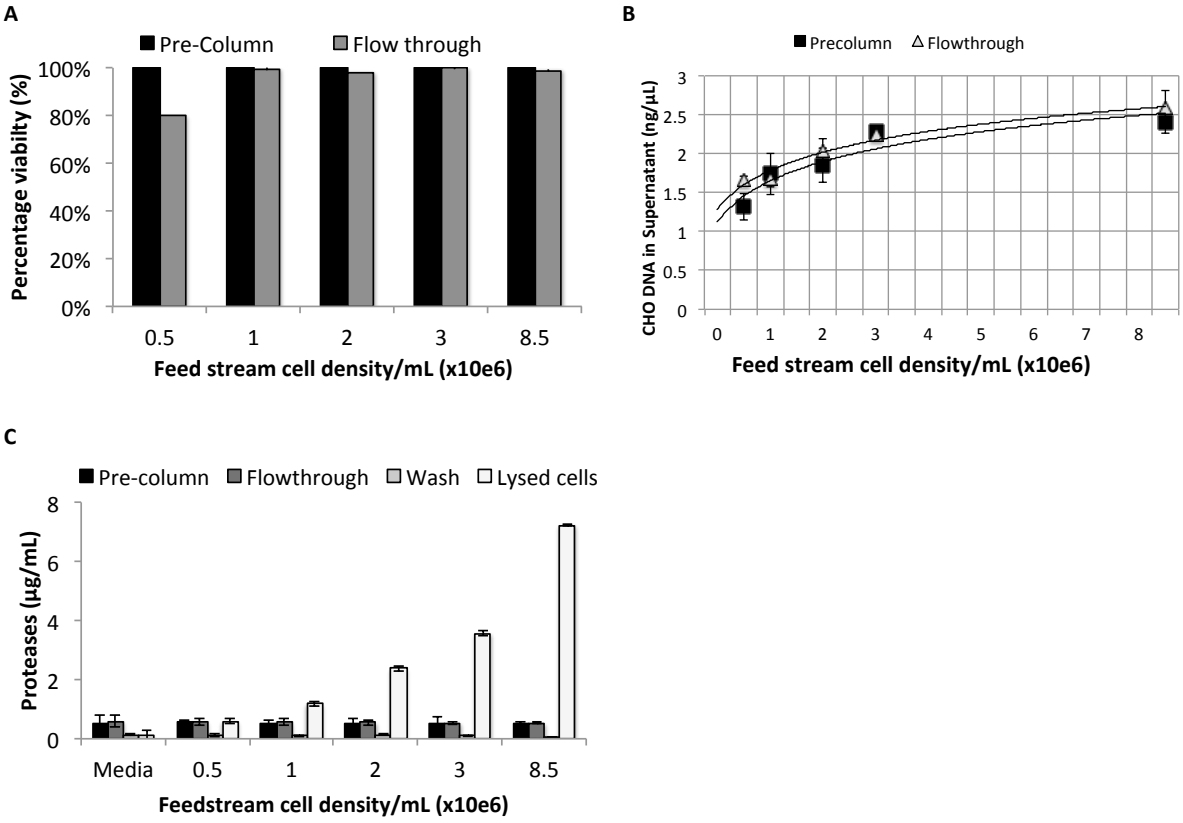

Figure 5.3 Analysis of unclarified feed streams containing different densities of CHO cells before and after passage through a scale down radial flow column with BigBead resin. CHO cells were cultured as described previously in shake flaks and re-suspended to a final cell density of 0.5, 1, 2, 3, or 8.5x10^6 cells/mL. The model feed streams were passed through the scale down column using the 200µm pore frits and flow rates of 1CV/min. Samples of each feed stream and a media only control were taken prior to and after column flow and the cell viability, CHO cell DNA and protease content of the feeds analysed. A) Percentage cell viability of unclarified feed stream in pre-column, flow through and washing conditions as determined by Trypan Blue exclusion. B) qPCR quantification of host cell DNA in pre-column and flow through conditions. C) Quantification of supernatant protease activity in unclarified feed stream and maximum cell lyses samples using a FRET assay (Life Technologies). Analysis of column flow through from different cell density showed that there was a small reduction in cell viability in the 0.5x10^6 cell/mL feed stream but no such reduction in the at higher densities. There was also no change in the amount of protease activity or host DNA in pre and post column samples. Figures show mean data and error bars one standard deviation (n=3).

Although cell density and viability were measured with using the Trypan blue assay this did not control for lysed cells or, importantly, any aggregated matter from cell lysis or resin particles escaping in the flow through. Cell integrity and aggregate presence was assayed via examination of the particle size distribution of feed streams pre and post column. Counts of particulate matter from cell lysis and protein aggregation (<6.7μm) and larger particles such as cell aggregates (>22μm) increased in proportion with cell density. There was no significant difference at cell densities of 0.5, 1, 2 and $8.5x10^6$ cells/mL. These data verified that CHO cells survived passage through the column without significant detrimental effects when feed with up to $8.5x10^6$ cells/mL was applied to the column.

Column washing is important to ensure the eluted product is as free of potential contaminants as possible prior to further down stream processing steps. In this case, as the solid-liquid separation steps are combined, there is potential for an increased amount of contamination as cells, the cause of most contaminating factors, are present during product capture. The column was washed with 10CV resulting in effective DNA clearance (zero detection), a decrease in proteolytic activity to <0.15 units/mL and a low particulate count (Figure 5.3, Figure 5.4). Furthermore, although cells were found in the first 2 column volumes of column washing there was complete clearance of cells by column volume 5. These results reinforced previous results showing that cell passage through the column did not create extreme shear environments that affected contamination of product, including at increased cell densities.

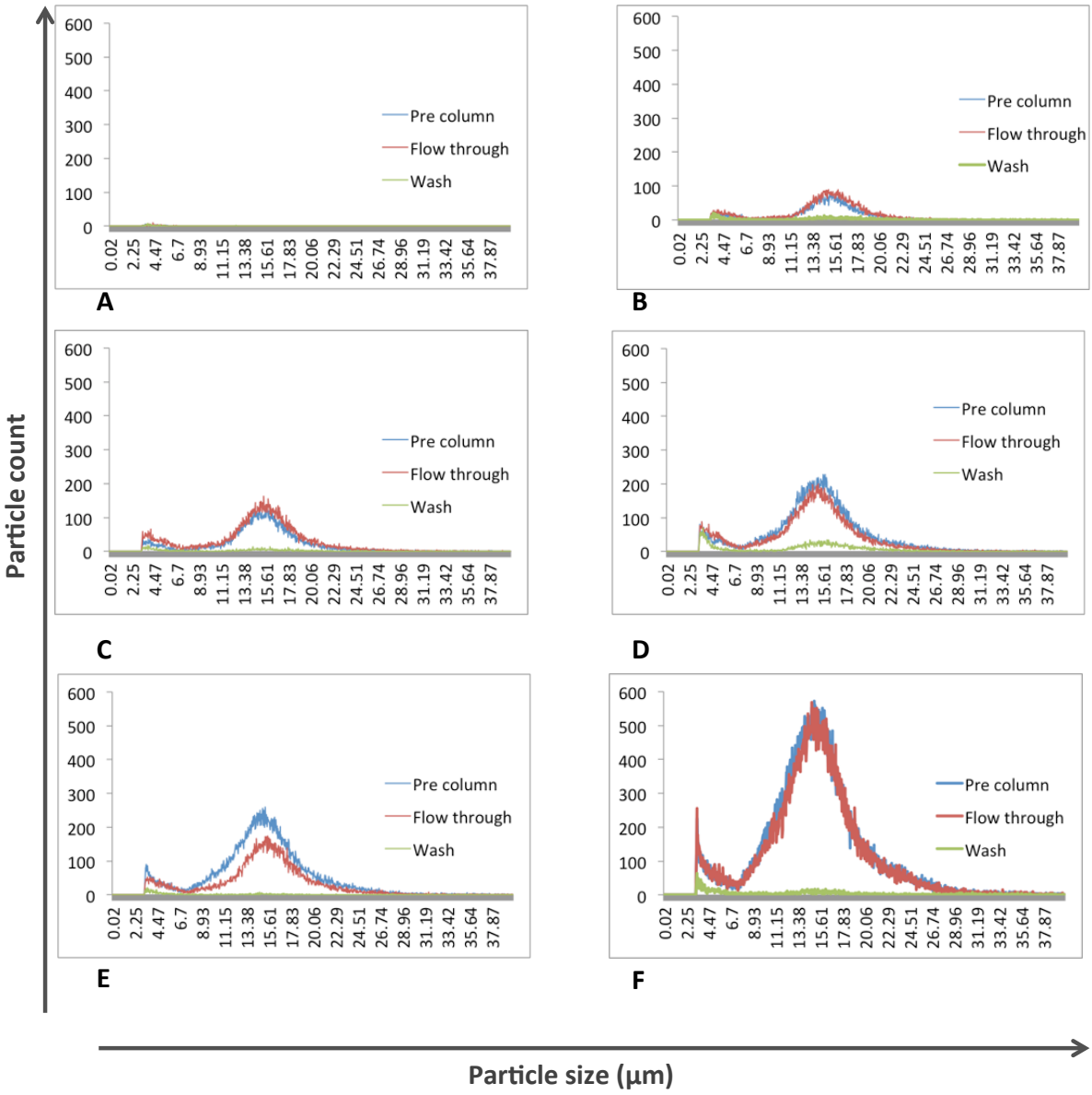

Figure 5.4 Particle size distribution of crude feed streams applied to a scale down radial-flow column packed with BigBead resin. CHO-S cells were cultured as described previously (**Figure 5.3**). Samples were removed from both pre and post-column feed streams as well as from column washes and the particle size distribution analysed. Feed stream cell density: A) Media only B) $0.5x10^6$ cells/mL C) $1x10^6$ cells/mL D) $2x10^6$ cells/mL E) $3x10^6$ cells/mL F) $8.5x10^6$ cells/mL. Blue indicates pre-column sample, red flow through and green PBS wash.

5.2.4 Model protein binding to IMAC resin in media modelling fermentation conditions

Recovery of His-tagged protein is dependent on the composition of media from which it is purified; accordingly, media is usually buffered to favour IMAC binding conditions prior to purification. In order to simulate IMAC capture at the end of CHO-S fermentation, a 12 day-old non-transfected shake flask culture that had been clarified and spiked with 1mg/mL His-tagged ScFv (His-SHMFE) was used to model binding of His tagged protein in buffered and non-buffered conditions. Protein was recovered using the scale down radial flow bed packed with large bead resin as previously discussed. Binding was analysed from both un-buffered and buffered media conditions using UV absorbance and SDS-PAGE. Un-buffered media received no addition prior to chromatography whilst buffered media was adjusted to 150mM NaCl, pH >7.5 and 20mM imidazole. Total product recovery was improved in buffered media, 83% versus 61%, as determined by absorbance at 280nm (data not shown). UV conductivity and SDS-PAGE resolution of protein captured from buffered media showed that the main protein fraction was eluted in CV3 and CV4. There was no visible protein band in the column flow through suggesting adequate binding to the IMAC matrix (Figure 5.5). The model protein was successfully recovered from media conditioned to simulate typical harvest conditions, showing that His-tag recovery was feasible using the combination of large bead resin and scaled down radial flow column.

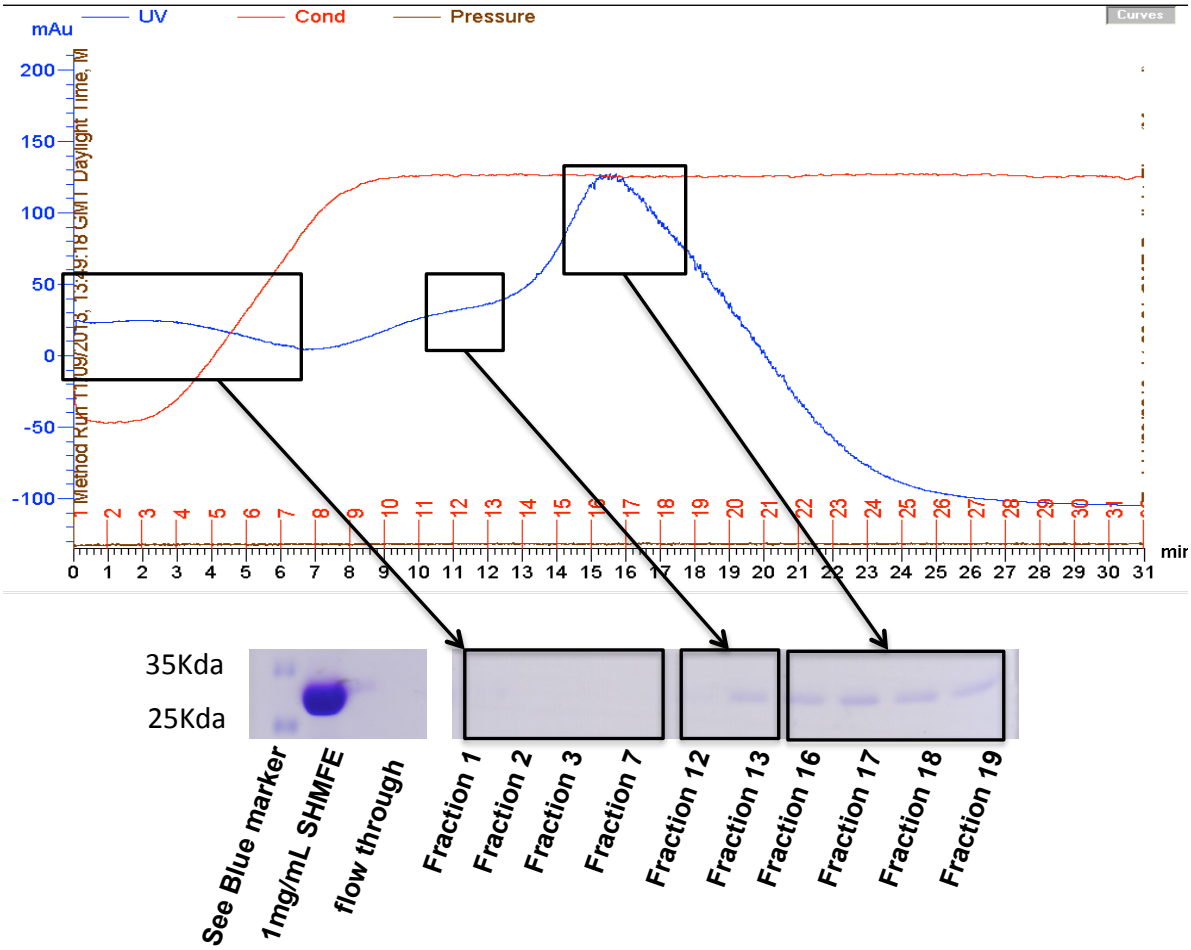

Figure 5.5 UV absorbance and SDS-PAGE resolution of eluted fractions of a model His-tagged single-chain variable fragment from conditioned media buffered to IMAC binding conditions. Simulated capture of a His-tagged protein from media at the end fermentation was achieved using spent growth media that was buffered to 150mM NaCl, pH >7.5 and 20mM imidazole and spiked with a model scFv (His6-tagged SHMFE) at a concentration of 1mg/mL. The media was run through the column at a rate of 1CV/min and capture by the large diameter IMAC resin was analysed by UV absorbance and SDS-PAGE resolution. Blue line indicates UV absorbance and red line shows conductivity (change from running buffer to elution buffer). Red numbers indicate 1mL fraction collection points. Results show that there is no protein band visible in the early fractions and a set of bands from fractions around the main peak of elution.

5.2.5 Buffer Optimisation for His-tagged CEA recovery

Having established that His-tagged proteins could be recovered from buffered media using the model protein, His-tagged CEA purification was optimized after expression in stably selected CHO cells. Column application buffer optimisation was performed in order to achieve maximum recovery of the target protein from clarified shake flask supernatant. There are a number of processes and buffers that would need to be optimized in the purification process for CEA (i.e. feeding strategy, column velocity and cell density). However the most relevant to this study was the column application buffer in order to improve recovery of expressed product without destroying cells. For instance cells exposed to excessive NaCl concentrations can undergo lysis and initiate protein denaturation due to the presence of proteolytic enzymes.

Three main factors were investigated as part of an experimental design incorporating a central composite, two-level, three-factorial optimisation to investigate the effects of pH, NaCl and imidazole concentration on product recovery using a 5mL HisTrap column. DesignExpert8 (Shukla et al., 2001) was used to determine the necessary experimental grid for optimising buffer composition. One significant factor interaction was found between pH and NaCl concentration ($P<0.05$) (Figure 5.6 A and B). Percentage recovery ranged between ~36%-73%. Reducing NaCl concentration at high pH and increasing it at lower pH showed equally improved recovery of CEA (Figure 5.6 C and D). The lower pH of 7.5 was selected for further experiments to reduce cell stress whilst imidazole was maintained at 20mM after no effect was found on CEA yield. However purification at pH 7.5 meant that a high concentration of NaCl was required (500mM) with potential negative effects on cell integrity.

pH	NaCl (mM)	Imidazole (mM)
8.5	500	10
8.5	500	30
7.5	500	10
7.5	111	10
8.5	111	30
8	305	20
7.5	111	30
8.5	111	10
8	305	20
7.5	500	30

Table 5.1 Design of experiments matrix for optimisation of IMAC binding buffer for capture of His-tagged CEA from unclarified CHO feed streams. IMAC buffer optimisation was carried out using DesingExpert8 to design an experimental matrix using a two-level, three factorial, central composite model.

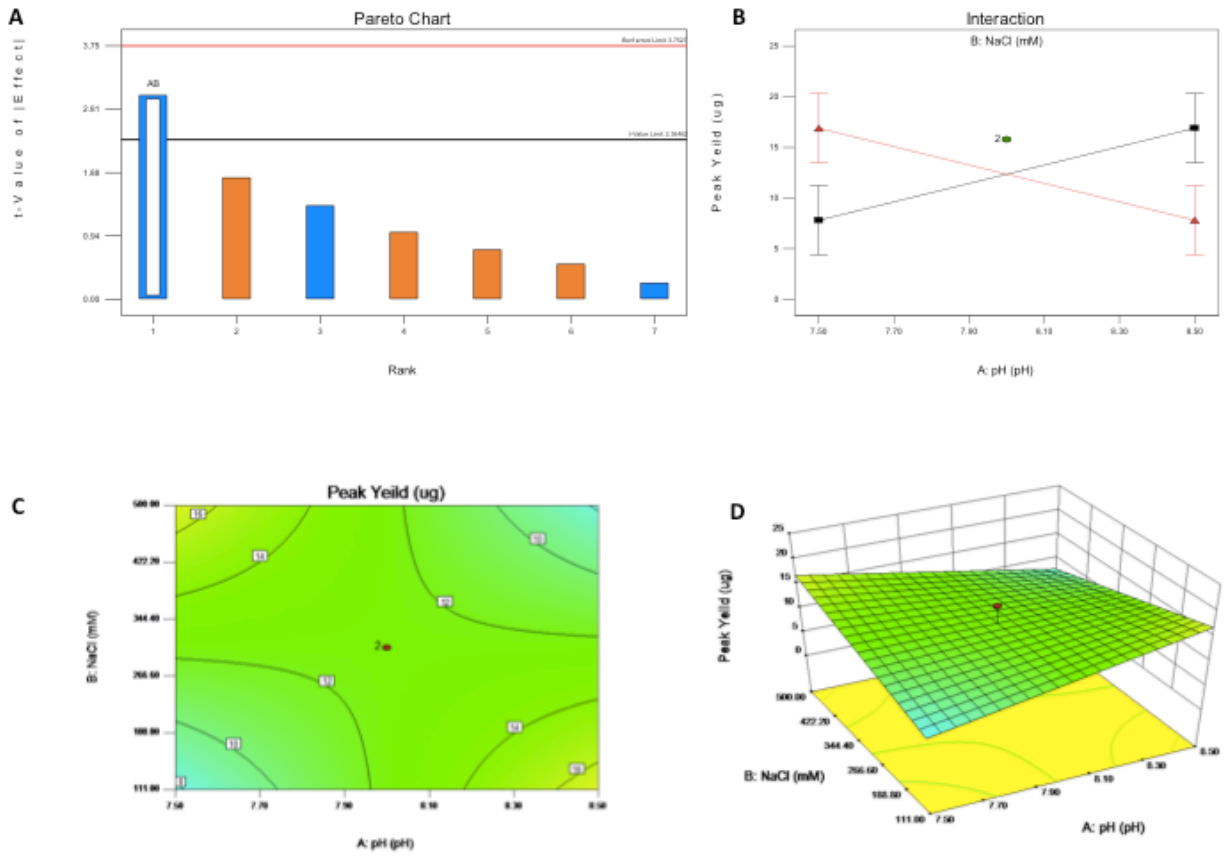

Figure 5.6 Design of Experiments based optimisation of buffer composition for IMAC capture of His-tagged CEA. CHO cells stably expressing His-tagged CEA were cultured in shake flasks to a density of $8x10^6$ cells/mL. Subsequently, the supernatant was centrifuged and filtered prior to buffering to the appropriate pH, imidazole and NaCl concentrations as specified in table 5.1. The buffered supernatant was passed through a 5mL HisTrap column, eluted and the protein capture analysed by ELISA. A) Pareto Chart indicates significant factor interactions above 1 value limit (black line). Blue indicates negative effects, orange positive effects and hollow significant effects (Factor labels; a = pH, b= NaCl, c= imidazole). B) Factor interaction of NaCl and pH (Red = 500mM NaCl, Black = no NaCl addition). C) 2D, and D) 3D surface response curve (heat map) of His-tagged CEA capture.

5.2.6 Cell survival under IMAC buffering conditions

Whilst buffer optimization had been shown to increase the percentage recovery of CEA from supernatant, the resulting exposure of cells to the high NaCl concentration required was anticipated to affect the viability and robustness.

The effect of high salt concentration and IMAC buffering on cell viability and feed characteristics were determined over an hour-long incubation. Viability of cells reduced by ~2% and 5% respectively in shake flasks with 200mM and 500mM NaCl, whilst feeds exposed to higher NaCl concentrations lost a much greater proportion of viable cells; up to 34% (Figure 5.7 A). Microscope observations indicated that above 750mM NaCl there was a build up cell aggregation as well as reduced viability. Consequently binding buffer composed of 500mM NaCl, pH 7.5 and 20mM imidazole was selected for use in further experiments. The impact of IMAC buffer on cell robustness during column passage was tested by passing CHO-S cells in buffered media through the column to determine the impact on column passage.

Percentage viability in the flow through remained >98% whilst host contaminants remained low, with protease activity < 1units/mL and 2ng/μL hDNA (Figure 5.7 B and C). Particle size distributions corroborated these results showing no significant difference ($P>0.05$) between pre-column and flow through samples (Figure 5.7 D) as seen previously with scFv. The results indicated that the optimised IMAC buffer did not substantially affect cell characteristics, nor negatively affect protease levels in the feed or eluted fractions.

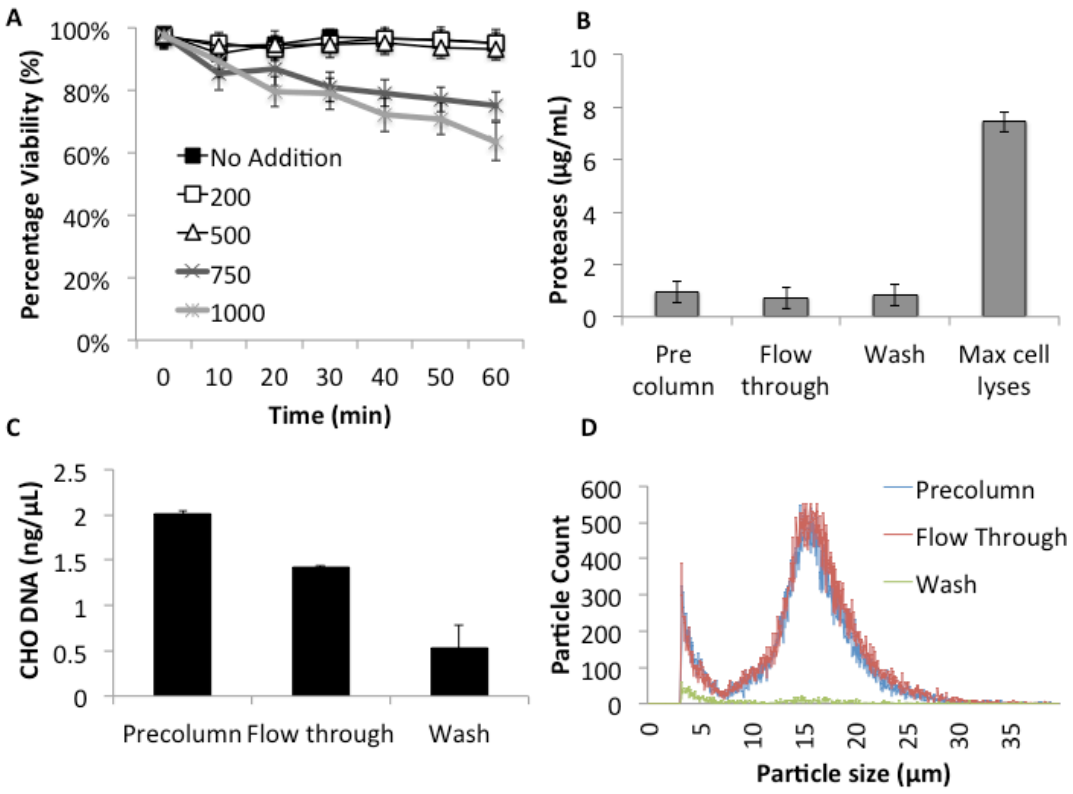

Figure 5.7 Cell survival and column flow characteristics of feed stream under optimised buffer conditions. CHO-S cells were cultured to 8.5x 10^6 cells/mL in shake flask cultures (>95% viability) before being buffered to the appropriate NaCl concentration in 300mL. A) The survival of CHO cells in buffered media with increasing NaCl concentration was analysed at 10-minute intervals over the course of 60min whilst being maintained under incubation conditions using an automated cell counter and Trypan Blue exclusion assay. The results showed that CHO-S cells survived up to 500mM NaCl with little effect on viability. B) Protease activity in unclarified CHO-S feed streams compared to maximum cell lyses samples after column flow buffered with 500mM NaCl, 20mM imidazole and pH 8.5. C) Host cell DNA in pre-column, flow through and wash samples cells passed through the radial flow column when buffered to optimum IMAC binding conditions. D) Particle size distribution (PSD) of feed stream passed through the column buffered with 500mM NaCl, 20mM imidazole and pH 8.5. These results show that the optimised IMAC binding buffer does not negatively affect the amount of host cell contaminants or amount of cell damage during column passage. Results show mean data with error bars one standard deviation (n=3).

5.2.7 Comparing recovery of His-tagged CEA from unclarified and clarified shake flask feed streams

The effect of media clarification on CEA-His recovery was investigated in the radial flow column and compared with recovery from a packed-bed, axial flow column (HisTrap). Recovery using the HisTrap column was hypothesised to be greater than the large bead IMAC resin in part due to the advantages of packed bed over large bead resin (i.e. greater surface area), however this column was used as a bench mark for comparison purposes.

CHO cells expressing CEA-His were cultivated in fed-batch shake flask conditions for 12 days (Figure 5.8 A). Feed was buffered and applied to the column either clarified or unclarified. Cells from the unclarified feed remained ~97% viable in column flow-through with low contamination levels of >1 unit/mL protease activity and >2ng/μL hDNA (Figure 5.8 B and C) and a particle size distribution that was indistinguishable from results with the scFv model protein (Figure 5.4). No difference in proteolytic activity was observed between unclarified and clarified feeds (Figure 5.8 B), however the hDNA quantity decreased in both clarified feeds, (1.95ng/μL from radial flow and 0.78ng/μL from HisTrap columns compared to 2.77ng/μL from unclarified feed). Protein recovery was higher from clarified media than from unclarified media (Figure 5.8 C), with the standard packed-bed HisTrap column achieving ~89% recovery, the radial flow ~78% and unclarified feed ~71% (Figure 5.8 D).

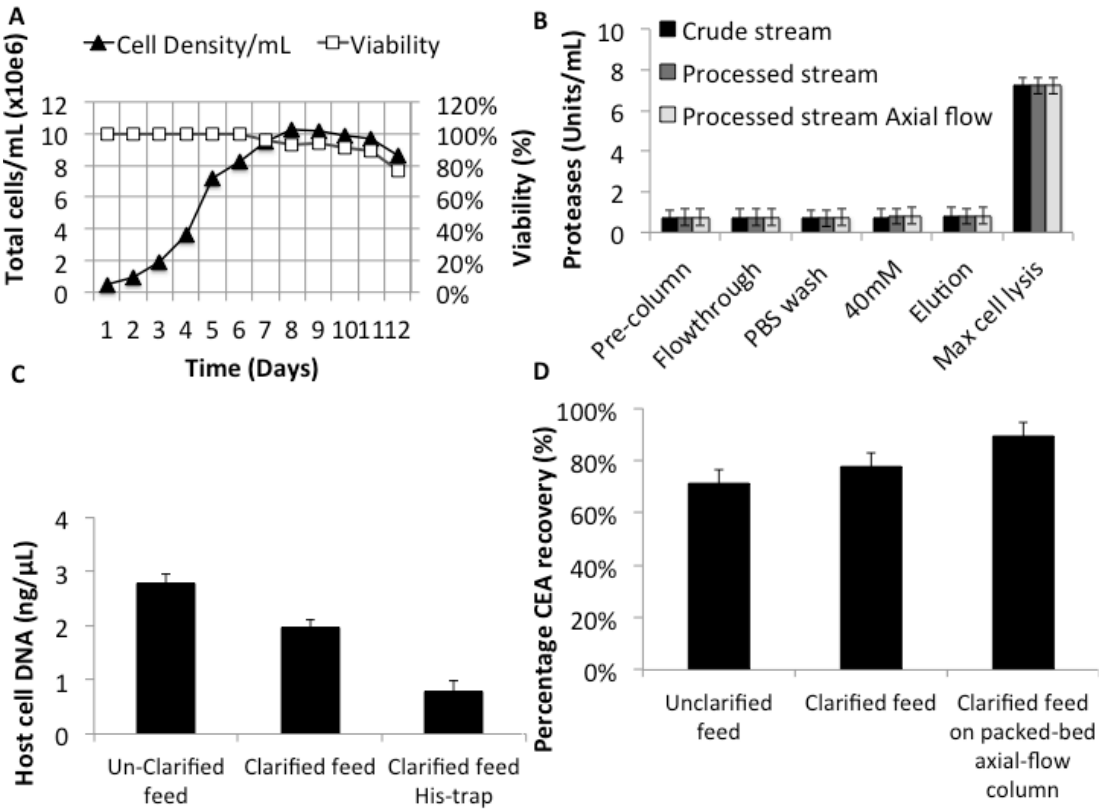

Figure 5.8 Comparison of crude and processed feed applied to RFC system packed with large diameter resin. HIS-CEA expressing CHO-S cells were cultured in shake flasks over a period of 12 days using fed-batch mode culture. The feed was separated into 3x300mL final volume and applied to the radial flow column in either an unclarified or clarified state or to an axial flow HisTrap column of the same volume after being buffered to optimised IMAC conditions or to the manufactures instructions. A) Fed-batch shake flask growth of CHO cells expressing His-tagged CEA. B) Viable cells/mL before and after passage through column. C) Protease activity in crude and processed feed streams after column flow through. D) Percentage recovery of His-tagged CEA from crude and processed feed streams as determined by ELISA. These results showed that recovery was improved in the HisTrap column as expected and that there was also an improved recovery in the radial flow column from clarified feed. Graphs show average data with error bars representing one standard deviation (n=3).

5.2.8 Generating a feed stream using bioreactor based fed-batch culture of CHO cells stably expressing recombinant CEA

A feed with increased volume, cell density and productivity was generated using a PBS3L single-use technology bioreactor. Cells were inoculated at a density of $0.3x10^6$ cell/mL with feed addition commencing 3 Days into fermentation. Once cells reached ~$10x10^6$ cells/mL the fermentation temperature was reduced to 35°c in order to improve glycoprotein expression and extend viability of the culture. The bioreactor feed had an increased cell density and product titre compared to shake flask growth ($11.8x10^6$ cells/mL and 7.2μg/mL CEA) (Figure 5.9 A and B).

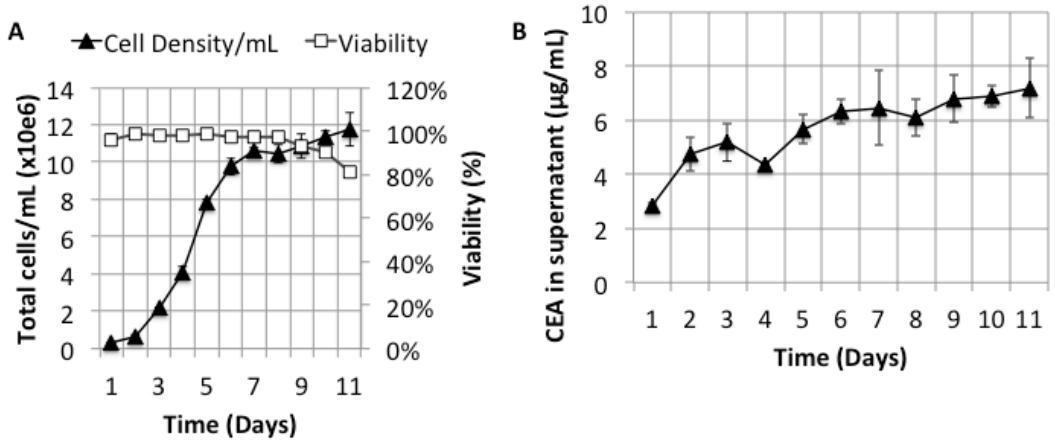

Figure 5.9 His-tagged CEA expression in a 3L fed-batch bioreactor fermentation. HIS-tagged CEA expressing CHO-S cells were cultured in a PBS-3L single use technology pneumatic bioreactor system (PBS Biotechnology, CA) under fed-batch conditions. Cells were seeded at $0.3x10^6$ cells/mL and reactor conditions set to 37°C, 25RPM vertical pneumatic wheel agitation, pH 7.2 and DO>30% via PID control. Feeding was started at 3% total volume (v/v) /day using CD-CHO Efficient Feed B as described previously after Day 4. On day 6 temperature and agitation were reduced to increase the length of fermentation and amount of glycosylated protein as described by (Nam et al., 2008). A) Fed batch fermentation of CEA-expressing CHO cells in SUT-3L PBS bioreactor. B) Time course of CEA expression in bioreactor fermentation as determined by ELISA.

5.2.9 Integrated recovery of His-tagged CEA via radial flow chromatography direct from bioreactor fermentation

A significant advantage of removing the requirement for solid liquid separation is that primary capture can be integrated directly into bioreactor harvest. As such a system of pumps and valves was designed to allow the multiple steps of primary capture to be carried out whilst the column remained *in situ* (see Figure 5.10). The inclusion of multiple buffer holding tanks and DSP process end points (i.e. elution, waste, washing and further DSP) enable simple charging, recovery and washing of the column.

Feed was harvested via passage directly through the column in 1L batches with subsequent regeneration to ensure consistency (see Methods 2.11). Cell viability and feed stream characteristics were not significantly different to previous experiments. Average product recovery was 69% (+/-6%) giving a final pooled sample yield of 14.6mg of His-tagged CEA as determined by ELISA. Fractions from individual purifications were diluted to 1μg/mL for Western blotting and were visualised using both anti-CEA and anti-His antibodies (Figure 5.11 C and D). There was no visible difference between subsequent purifications. CEA, but no His-tag, was identified in flow-through from the column indicating that His-tag recovery was efficient and the apparent lower yield compared to equivalent shake flask material was due to untagged or degraded CEA passing through the column.

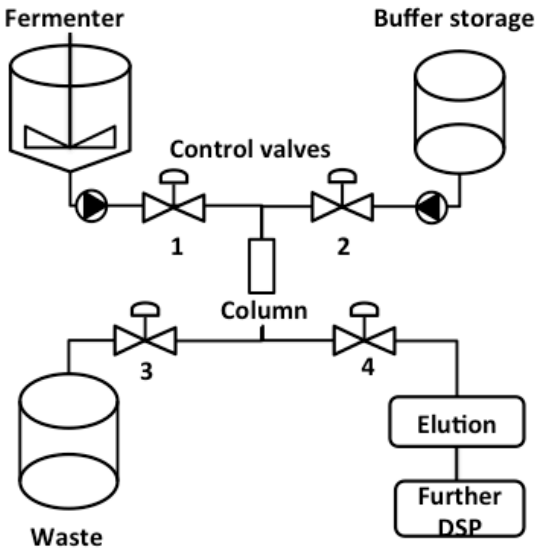

Figure 5.10 Process flow diagram of integrated radial flow chromatography control system. Integration of the radial flow system directly into the reactor harvest line was achieved using a combination of buffer storage tanks, pumps and control valves. Primary harvesting of the reactor in 1L batches (valves 1 and 3 open) was followed by washing (valves 2 and 3 open) and then elution (valves 2 and 4 open). Integration of the radial flow unit was carried out using a sterile Masterflex C-FLEX tube-welding unit to allow for cell culture to remain sterile at all times.

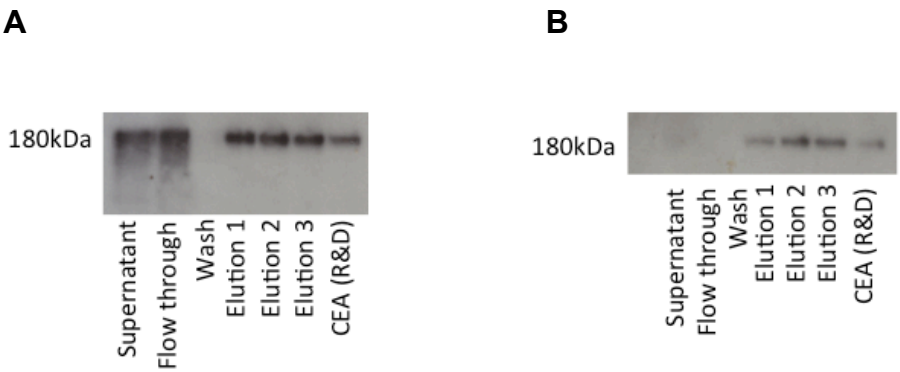

Figure 5.11 Western blotting of eluted fractions from reactor purification via radial flow chromatography. Samples removed from the bioreactor culture, column flow through and elutions were probed using an anti-CEA antibody (made in house) or an anti-HIS antibody (Qiagen, Netherlands) in order to asses the purity and success of CEA capture before both antibodies were detected using an HRP-conjugated anti-mouse antibody (R&D, UK). A) Anti-CEA western blot of radial flow column eluate. B) Anti-His western blot of radial flow column eluate. All elutions and positive controls were diluted to 1μg/mL prior to western blotting. Elution refers to separate experiments NOT fractions. The results suggest that whilst there is a substantial amount of CEA remaining in the flow through after column passage there is a good concentration rate of the HIS tag from the supernatant. Neither figure shows any staining activity in washing samples.

5.3 Discussion

The aim of this chapter was to develop and evaluate a system for primary recovery of recombinant proteins from unclarified CHO cell feed streams. Primary recovery techniques for recombinant proteins expressed in mammalian cells currently requires laborious solid-liquid separation prior to chromatographic capture. This process has been estimated to account for between 50-80% of the cost of production for some proteins (Levy et al., 2014). Process efficiency can be achieved by the better integration of steps directly following the bioreactor stage as these currently have large impacts on cost (Warikoo et al., 2012), with the added benefit of reducing process volumes, CAPEX, operator costs and consumables.

In this chapter an integrated primary capture step for recovery of secreted, recombinant proteins from unclarified mammalian cell feeds was developed using scale-down radial flow chromatography operated with large diameter resins.

Previous chapters in this thesis have focussed on methods for rapid production of an Fc conjugated scFv (scFv-Fc), however here the target antigen from previous chapters, recombinant HIS-tagged CEA, was chosen to illustrate the procedure. CEA is a heavily glycosylated molecule that is up regulated and secreted in a number of cancers and is therefore of interest as a therapeutic target of interest. CEA has previously been produced either by recovery from ascetic fluids (Hammarström, 1999., Sigma Aldrich) or in the yeast *Pichia pastoris*. However yeast expression has only achieved truncated forms of the protein (Hellwig et al., 1999; Sainz-Pastor et al., 2006; Tolner et al., 2013). Consequently CEA remains an expensive protein to work with requiring expression in mammalian or glyco-engineered insect cells to ensure correct glycosylation for structure and function.

As with other proteins that lack a natural purification tag primary capture can be facilitated by the addition of an affinity based capture method. The application of a His fusion tag is a simple and convenient purification technique for many proteins including CEA and IMAC capture offers a comparatively inexpensive, robust and versatile alternative to protein A/G based chromatography (Young et al., 2012). Whilst concerns have been raised about the potential of therapeutically redundant

purification tags to affect *in vivo* half life and therapuetic function of proteins, many tags are cleavable post-purification (Waugh, 2011) and His tags have been used in a number of clinical products without adverse effects (Tolner et al., 2013, Table 37.1).

The development of the integrated process discussed here took into account a number of feed stream factors that could have affected the performance of future process steps and recovery. Cell damage plays an important role in the characteristics of feed streams (Anspach et al., 1999; Salte et al., 2006) and has been shown to vary widely across bioprocess operations (Hogwood et al., 2013; Kim et al., 2013). Here the focus was particularly on proteolytic activity and hDNA contamination. Proteases directly affect product stability and affinity tag cleavage (Gao et al., 2011) and hDNA contamination significantly affects feed viscosity (Balasundaram et al., 2009) and must be at minimal levels to meet regulatory standards (Nissom, 2007). Previous studies in the area have focussed on host cell protein (HCP) content however this is not directly linked to product degradation (Cordoba et al., 2005).

In this process, cell damage was predicted to occur during column passage, especially as the cells traversed the relatively high shear regions of the porous frit. The results showed between 2-4% loss of viability and no significant difference (p= <0.05) between particle size distribution, protease activity or hDNA content in pre and post column samples. By comparison, previous results of direct capture using expanded bed adsorption (EBA) from hybridoma cells showed an increase in hDNA contamination of up to 54% (Feuser et al., 1999).

The current benchmark for primary protein recovery is axial packed-bed chromatography (as used in most industrial operations); these results demonstrated that percentage recovery of CEA was 17.9% greater in an axial packed-bed column (89.16%) compared to capture from unclarified feed using the large diameter resin in radial flow format (71.6%). In industrial processes His-tag recovery can be as high as 90% (Saraswat et al., 2013). In this chapter three factors (pH, imidazole, and NaCl concentration) were optimised for product capture, therefore a broader optimisation of process factors (bead diameter, bed height, load mass, flow rate, cell density, temperature etc) would be expected to improve yield.

5.3.1 Conclusions

This chapter shows that primary recovery of recombinant proteins from unclarified media is a functional processing technique that can integrate easily with current bioprocess tools. Whilst the data presented here demonstrates primary capture of a heavily glycosylated, His-tagged CEA molecule, the generic use of CHO cells as an expression host suggests that this approach is applicable to the recovery of many different proteins using other modes of chromatographic interactions.

*The work presented in this chapter has been published as; Kinna et al., 2015

Chapter 6 Conclusions and future work

This chapter aims to review and summarise the results of this thesis and provide recommendations for the future direction of related work.

6.1 Development of a rapid and cost effective transient gene expression system in mammalian cells for use in lab scale production

The use of transient expression in mammalian cells to produce recombinant proteins at small scale is well documented (Durocher et al., 2002; Wurm, 2004; Ye et al., 2009; Longo et al., 2013). However, transient expression systems can be cumbersome and difficult to scale in order to meet increased demand. They can also be prohibitively expensive in the small lab environment leading to submaximal screening. In chapter 3 a recombinant protein expression system was developed and optimised for rapid, scalable and cost effective production of recombinant proteins in suspension adapted 293 cells. The system was used to express the first quantities of a model scFv-Fc fusion protein targeted against CEA, which were then characterised with an aim to further develop it.

ScFv-Fc constructs are encoded on a single plasmid and therefore make good model proteins for investigating transfection and expression unlike proteins that require co-transfection of multiple plasmids, such as the light and heavy chains of an antibody (Pybus et al., 2014a), which adds a further complication in studying transfection efficiency and expression.

Preliminary transfection efficiency data generated using fluorescent reporter proteins showed that the HEK293F cell line was transfected more efficiently with PEI than CHO cell lines contributing to greater protein expression. The cytotoxic effects of transfection has been shown previously (Jain et al., 2013), and this was evident in all transfection conditions investigated here. As high transfection efficiency plays a significant role in transient gene expression the combination of the HEK293 cell line and PEI reagent was optimised in terms of DNA: PEI ratios to improve expression. Previous optimisations of PEI to DNA ratios in transfection of cell lines have varied from 1:1 to 5:1 dependant on cell lines (Fang and Shen, 2010; Thompson et al., 2012; Xie et al., 2013; Longo et al., 2013), however these figures may differ significantly

based on batch-batch variation when PEI solutions are made in-house and could therefore require optimisation on a 'per-batch' basis. The results presented here show that the optimum ratio was 2:1 (PEI:DNA) for transfections. Using linear 25 kDa PEI solutions made in-house cost in the region of £0.70/mL compared to £405.00/mL for the commercial reagents tested and is used in similar quantities during transfection (prices correct as of Feb 2016 – suppliers Polyscience, Germany and ThermoFisher Scientific, UK).

Scale up of transient gene expression was carried out using a linear 10-fold increase of culture volume and transfection solution from 30mL-300mL-3L including the use of shake flasks and a pneumatic SUT bioreactor. Scale-able transient transfection has been carried out using a number of methods including transfection and electroporation of high cell density cultures prior to expansion (Backliwal et al., 2008b; Baldi et al., 2007) and linear increases in transfection solutions such as those used in most commercial kit reagents. The results shown here indicated that there was an improvement in yield at a scale of 300mL, however all conditions were comparable in terms of cell growth and protein yield. The simplicity of a linear increase in transfection solution and no requirement for further process optimisation at increased capacity are the main advantage of this scaling method compared to a number of other techniques.

Stability characterisation of scFv-Fc produced using the different scales and reagent combinations tested showed there was no difference in the quality of the proteins. The DSF assay is now well established in determining and comparing stability between similar proteins including those with aggregation potentials and is also routinely used in screening ligand interactions involved with protein stability (Niesen et al., 2007; Menzen and Friess, 2013). DSF has been directly compared with differential scanning calorimetry (Wen et al., 2012; Shi et al., 2013) showing comparable results, however the DSF assay does not carry the cost of DSC in equipment outlay, time or protein required for the assay. Combined with more standard protein stability analyses techniques the profile presented suggests that the protein was highly stable.

The scFv-Fc was also analysed by surface plasmin resonance in a Biacore T200 resulting in very high estimations of binding affinity. This was confirmed by *in vitro*

binding indicating that the construct had both a high affinity and selectivity for CEA. The results shown contributed significantly to continued development of this protein candidate and to methods used for the production of a number of other recombinant proteins.

The cost of commercial transfection reagents and protein analysis assays can prove limiting when factors such as scale and multiple transfections are considered, especially in the small lab environment. This work shows that comparatively inexpensive reagents and techniques can be used to great effect without reducing quality of results or productivity of recombinant proteins.

6.2 Stable integration and expression of SM3EL-Fc in CHO-S cells and comparison of product stability

The scFv-Fc investigated in chapter 3 produced promising results during characterisation. A production system with greater capacity was required to increase yield and further reduce costs for larger quantities of recombinant protein for further investigation. In chapter 4 methods to rapidly obtain a stably expressing population to increase protein yield were assessed.

During characterisation and preclinical phases of development clonal populations are not necessary unlike for clinical grade use. Whilst heterogeneity should be avoided when characterising products, there is a level of heterogeneity even in clonally produced proteins (Pilbrough et al., 2009). The application of stable cell based protein expression to rapid protein production has been discussed previously (Ye et al., 2010), as has the difference in transient and stably expressed glycoproteins (Ye et al., 2009) suggesting that there is no difference in the activity of proteins due to their glycoprofiles after expression in different systems. Stable cell pools used in early production stages provide all the benefits of stable cell expression as well as reduce mass DNA production and variability between transfections.

Two DNA integration systems designed to increase expression in stable cells and to allow for rapid selection and cell line developments were investigated in chapter 4. Whilst flp-mediated stable cell generation was unsuccessful, cloning the model scFv-Fc into a UCOE expression vector did allow for stable cell selection. Isogenic cell

technologies have generally been used for research purposes as opposed to production in the literature, however site-specific recombination has been used for high expressing cell line generation (Codamo et al., 2011) Unlike flp-mediated integration, which yields an isogenic population, expression using the UCOE vector produces a variable population. However any variability in genomic insertions does not negatively effect the characterisation or activity of the protein during early stage development (Ye et al., 2010). Similar vector-based techniques for ensuring active chromatin regions at transgene insertions include scaffold/matrix (S/MAR) and, alternatively, bacterial artificial chromosomes (BACs) (Kunert and Casanova, 2013).

Comparing transiently expressed protein, which has associated heterogeneity, with protein from stable cell pools showed no difference in stability or effector function in this study. Previous comparisons of protein activity have often focussed on differences between transient and stably produced proteins or HEK293 vs. CHO expressed proteins (Geisse and Voedisch, 2012). These results indicate that characterisation data from transiently expressed protein in HEK293 cells is directly comparable to stably produced protein using non-clonal CHO populations and that stable cell pools can be used effectively in the production of recombinant protein for early development.

6.3 Primary recovery of recombinant proteins from unclarified mammalian cell feed streams using radial flow chromatography and large bead IMAC resin.

Primary capture of recombinant proteins from mammalian cells is both a costly and lengthy process, as discussed in chapters 1 and 5. Here this challenge has been addressed by developing an integrated primary capture step for recovery of secreted, His-tagged recombinant proteins from unclarified mammalian cell feeds using RFC operated with large diameter resins. As yield is a function of the number of processes (Farid, 2011), it is advantageous to remove process steps where possible.

The improvement in process efficiency yielded from using an integrated capture step would result in a reduction in COG/g and has been shown previously with capture techniques from unclarified feeds (Schügerl and Hubbuch, 2005). EBA, a format with similar process advantages to large-bead resins, has been compared with standard

bioprocess techniques resulting in reduced COG/g and simplified recovery in microbial and mammalian cell downstream processing (Willoughby et al., 2004; Chhatre et al., 2006; Tolner et al., 2013). Similar efficiency gains from the removal of clarification steps could be expected with large bead diameter resins. Uptake of novel techniques can be affected by concerns over regulatory compliance however the combination of RFC and EBA has been applied to unclarified microbial fermentation broths using His-tagged proteins previously in cGMP-compliant processes (Tolner et al., 2013), showing that adoption of similar approaches does not necessarily introduce regulatory hurdles.

In this thesis the radial flow column has been used with maximum volumes of 1L batches and a viable cell density of ~$12x10^6$ cells/mL whilst fully integrated into the harvest system. Industrial feed volumes, cell densities and protein titers can far exceed those used here however ultimately the data presented here shows that there is potential for this system to be integrated into larger scale production chains after optimization. Whilst further work must be carried out in order to investigate the effects of factors such as cell density and protein concentration as well as flow velocity on product capture, this primary capture technique has obvious industrial applications given that it can reduce the number of processing steps and volumes involved in protein purification potentially reducing costs and time for protein recovery at many scales.

6.4 Reducing the cost of recombinant protein expression and purification

A key part of each of the chapters in this thesis has been to underline the cost effectiveness of the techniques used.

Transient gene expression enables production of recombinant proteins at a far greater speed without the associated costs and time of stable cell selection. Transiently expressed proteins can also predict the behaviour of those from stable expression saving costs on screening proteins. Using PEI, a significantly cheaper transfection reagent than those in commercially available kits, reduced the cost of transfection and is available as a GMP compliant reagent permitting its use in early and late stage clinically relevant transfection.

Stable cell based expression using a UCOE expression facilitated larger scale production of recombinant protein without the need to produce DNA and transfect on multiple occasions. This simulated selection of a lead candidate after screening many proteins by transient expression. In order to produce large quantities of protein, high expressing stably selected cells and an optimised feeding and growth strategy proved the most efficient production method.

Chromatographic capture of proteins and primary recovery is often the most expensive part of protein separation. In chapter 5 a method is proposed that can remove the need for solid liquid separation entirely saving all associated costs. Some estimates put this cost as high as 80% of total process cost that could be partially saved.

The methods for production, purification and analysis of recombinant proteins in suspension adapted HEK293 and CHO cell lines discussed here can be used by many labs engaged in early stage screening of drug candidates. The protocols and techniques have been selected to address the necessity for rapid, simple and inexpensive production at lab and preclinical stages of development and can therefore form a methodology for those aiming to complete similar projects.

6.5 Future work

The results presented in this thesis show that a simple, cost effective and rapid expression system can be used to produce sufficient recombinant proteins (both antigen target and targeting antibody) for early screening of novel drugs. Characterisation of proteins from transient expression in HEK293 cells provided a reliable platform for the selection of candidates of interest and subsequent scale up of production.

Whilst the yield of the model scFv-Fc construct used here was sufficient for characterisation and further study, there is significant variability in the expression of even highly similar proteins. Hence, more difficult to express proteins can require further improvement to obtain sufficient protein expression. This can sometimes be

achieved using engineered tags to improve expression and solubility levels of the target (Young 2012). Glycosylation of recombinant proteins and its importance has been discussed here however was not analysed as part of this data, future work should include analysis of the variability of these systems in terms of glycosylation effect.

Recombinant protein expression has been shown to increase when rates of cellular division are inhibited, either to slow it or to halt it (Dietmair et al., 2012; Dinnis and James, 2005). Techniques that have been used to induce a stalled cell cycle include; nutrient limitation, reduced pH, reduced temperature or hyper osmotic pressure (Dinnis and James, 2005) as well as cell cycle inhibitors, such as the P53 regulated-Gadd45 protein (Kim et al., 2014, p. 45) or P21/27 (Ibarra et al., 2003), in combination with cells adapted to high cell density culture. Cell cycle control techniques have also been used in combination with electroporation methods as well as lipofection based methods (Golzio et al., 2002).

Biphasic (two phased) culture of cells uses these findings to create a high cell density in the first phase of growth with rapid cell division, followed by a secondary phase with division inhibited but growth enhanced. Thus the primary growth stage is shorter than usual and the secondary production phase enables a greater titre than single-phase growth. Research has been carried out that incorporates numerous methods for increasing expression including those discussed above to great effect (Codamo et al., 2011).

Further work may include establishing a simple method to transfect cells at an increased cell density and optimisation of the recovery time prior to cell cycle inhibition. Of particular interest is reduction in temperature to induce cell cycle inhibition as this represents the lowest costs.

Other reported methods to increase transfection efficiency include synchronisation of cell cultures prior to transfection as discussed by Grosjean et al (2002) and since in some commercial kits. This enables the addition of the transgenic DNA within the optimum cell cycle stage for efficient transfection yet has not been widely revisited These techniques alone or in combination may well provide further increases in transfection efficiency for both transient and stable based protein expression.

The use of transiently expressed scFv-Fc has been shown here to be a good model of stably expressed product however; the variable expression performance of numerous protein scaffolds suggests that this may not be true for all proteins. It would therefore be advantageous to characterise the effects of cross-cell and expression type variation based on protein scaffold.

In chapter 5 a technique was developed to capture His-tagged protein from unclarified mammalian cell feed streams. The process utilised IDA chelating agarose beads of between 300-500μm in diameter forming a packed resin charged with nickel. However, a range of resins are available differing in construction, functional groups, bead size and packing density (Hochulial., 1987; Bornhorst and Falke, 2000; Warren and Bettadapura, 2005; Saraswat et al., 2013). These variables are expected to have a significant impact on performance and feed stream characteristics e.g. bead diameter will directly affect interparticle porosity affecting cell travel through the column, fluid dynamics and the shear rate experienced by the cells thus affecting protein yield.

Initially a protein A based resin was imagined for capturing proteins from unclarified feeds, however its associated costs proved prohibitive. It would therefore be worthy of further investigation for the recovery of high value Fc containing proteins which make up a huge portion of the biopharmaceuticals industry. Whilst HIS-tag based capture has been used in GMP settings, protein A based capture has the advantage of being routinely used in GMP compliant processes. This being said, IMAC capture of proteins with naturally occurring histidine rich regions has also been demonstrated, including in antibodies and antibody fragments (Todorova-Balvay et al., 2004; Cheung et al., 2012). Hence, another area for further investigation could be the possibility of utilising comparatively inexpensive IMAC resin for large scale primary capture of antibody based molecules prior to polishing using the more expensive protein A resin.

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Appendices 1

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CLUSTAL 2.1 multiple sequence alignment


ShMFE23         --QVKLEQSGAEVVKPGASVKLSCKASGFNIKDSYMHWLRQGPGQRLEWIGWIDPENGDT 58
ShMFE           --QVKLEQSGAEVVKPGASVKLSCKASGFNIKDSYMHWLRQGPGQRLEWIGWIDPENGDT 58
SM3E            MAQVKLEQSGAEVVKPGASVKLSCKASGFNIKDSYMHWLRQGPGQCLEWIGWIDPENGDT 60
                  **************************************** **************

ShMFE23         EYAPKFQGKATFTTDTSANTAYLGLSSLRPEDTAVYYCNEGTPTGPYYFDYWGQGTLVTV 118
ShMFE           EYAPKFQGKATFTTDTSANTAYLGLSSLRPEDTAVYYCNEGTPTGPYYFDYWGQGTLVTV 118
SM3E            EYAPKFQGKATFTTDTSANTAYLGLSSLRPEDTAVYYCNEGTPTGPYYFDYWGQGTLVTV 120
                ************************************************************

ShMFE23         SSGGGGSGGGGSGGGGS-----ENVLTQSPSSMSVSVGDRVTIACSASSSVPYMHWLQQK 173
ShMFE           SSGGGGSGGGGSGGGGS-----ENVLTQSPSSMSASVGDRVTIACSASSSVPYMHWFQQK 173
SM3E            SSGGGGSGGGGSGGGGSGGGGSENVLTQSPSSMSVSVGDRVTIACSASSSVPYMHWLQQK 180
                **********     ***********.*********************:***

ShMFE23         PGKSPKLLIYLTSNLASGVPSRFSGSGSGTDYSLTISSVQPEDAATYYCQQRSSYPLTFG 233
ShMFE           PGKSPKLLIYSTSNLASGVPSRFSGSGSGTDYSLTISSVQPEDAATYYCQQRSSYPLTFG 233
SM3E            PGKSPKLLIYLTSNLASGVPSRFSGSGSGTDYSLTISSVQPEDAATYYCQQRSSYPLTFG 240
                ********** ************************************************

ShMFE23         GGTKLEIKAAA--------------- 244
ShMFE           GGTKLEIKAAA--------------- 244
SM3E            CGTKLEIKRSPRGPTIKPCPPCKCPA 266
                 ******* :.
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Cluster alignment of 3 affinity matured scFv constructs targeting CEA. shMFE23 – humanised version of MFE23, shMFE- shmfe23 with further stabilising modifications, SM3E stabilised and humanised with G4S linker.

Appendices 2 – Kinna *et al.,* 2015 manuscript